GEOGRAPHIC CHARACTERIZATION AND ENVIRONMENTAL DETECTION OF *Neoparamoeba perurans* THE CAUSATIVE AGENT OF AMOEBIC GILL DISEASE

by

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DEDICATION

I dedicate this thesis to my wild and crazy family. It has been hard being away from all of you for so long but knowing that you were rooting for me to follow my dreams and have my adventures has meant the world to me. Lisa, having you to talk to about health, has kept me grounded and focused on loving myself. Stewart, knowing that you had the important things covered back home was the greatest gift you could have ever given me. Matthew and Caragh thank you for keeping me entertained with stories from your lives and your crazy conspiracy theories! To my grandparents who passed away while I was completing this degree, thank you for the love and support. Thank you for helping to inspire my love of life, nature and adventure that launched me on this path. Mom, I cannot thank you enough for the love and support you have given me. You are my rock and I could never have done any of this without you!

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DECLARATIONS AND STATEMENTS

Declaration of Originality

This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright.

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ABSTRACT

Amoebic gill disease (AGD) is a global disease that has major effects on Atlantic salmon aquaculture. AGD is caused by the facultative marine amoeba, *Neoparamoeba perurans.* The disease first appeared in farmed salmon in Washington and in Tasmania in the mid-1980s. Since then, AGD has become a cosmopolitan problem with an increasing mortality and economic impact. Outbreaks of AGD have been reported in thirteen countries across six continents. Even where gross pathology is not evident, there is PCR evidence that *N. perurans* is present on salmon gills in other geographic regions, thus creating the potential for further outbreaks. This research aimed at understanding the relationship between geographically diverse amoeba populations isolated from the gill of infected salmon, the parasite and the environment. This improved understanding of these relationships could help inform industry decisions associated with management of AGD associated risks. With respect to understanding the geographical relationships, PCR-based typing methods were used to compare samples obtained from Australia, Canada, Ireland, Scotland, Norway, and the United States of America (Chapter 2 and 3). Sequences of highly conserved genes were compared using Multilocus sequence typing (MLST) (Chapter 2), to create a dendrogram showing the relationships between samples. The analysis resolved low-level differences between samples. Building upon these analysis (Chapter 2), the Random Amplified Polymorphic DNA (RAPD) technique was used (Chapter 3) to help elucidate the extent of the genetic differences that were observed (Chapter 2). RAPD allowed for a better understanding of the total genetic differences contained within the isolate genomes. The analysis showed high polymorphisms between samples. Though *N. perurans* is a ubiquitous organism, the apparent population differences (Chapter 2 and 3) may indicate the existence

of localized populations not associated with fish. Two methods were utilized to investigate the presence of *N. perurans* within benthic sediments (Chapter 4). These methods were further applied at two locations (Canada and Tasmania) containing commercial farms. *N. perurans* was present in the sediment at both locations indicating the potential of sediment as a reservoir. Finally, Chapter 5 concludes the findings of this research and relates them to the current knowledge of the *N. perurans,* its role in the environment and potential for risk management.

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LIST OF ABBREVIATIONS

°C	Degrees centigrade
AGD	Amoebic Gill Disease
AUS	Australia
AUD	Australian Dollar
bp	Base pair
BKD	Bacterial Kidney Disease
BURST	Based Upon Related Sequence Types
CAN	Canada
cm^2	Centimetre squared
CV	Coefficient of Variation
DAPI	4',6-diamidino-2-phenylindole [fluorescent stain]
DIC	Differential Interface Contrast
DFO	Department of Fisheries and Oceans
DNA	Deoxyribose Nucleic Acid
dNTP	Deoxyribonucleotide triphosphate (ATCG)
DP	Discriminatory Power
EDTA	Ethylenediaminetetraacetic acid
ERM	Enteric Redmouth
FAO	Food and Agriculture Organisation
GC	guanine-cytosine
GPS	Global positioning system
h	Hours
IFAT	Immunofluorescent antibody test
IHN	Infectious Hematopoietic Necrosis
IHNV	*Rhabdovirus*
ISA	Infectious Salmon Anaemia
IPN	Infectious Pancreatic Necrosis
ITS	Internal Transcribed spacer
kb	kilobit
L	Litre
LOD	Limit of Detection
LOQ	Limit of Quantification
NaCl	Sodium Chloride (salt)
nM	Nano molar
N/A	Not Applicable
N/D	No Data
No.	Number
NSW	New South Wales
m	Meter
m^2	Meter squared
M	Molar
mM	Millimolar
mm^3	Millimetre cubed

min	Minutes
mL	Millilitre
mg	Miligram
MLST	Multilocus Sequence Typing
MYA	Malt Yeast Agar
MYS	Malt Yeast Seawater
PCR	Polymerase Chain Reaction
PD	Pancreas Disease
PFGE	Pulsed-Field Gel Electrophoresis
pH	potential of hydrogen – acidity measure
ppt	Parts per thousand
PRV/HSMI	Piscine orthoreovirus
qPCR	Quantitative Polymerase Chain Reaction
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Length Polymorphism
RNA	Ribonucleic Acid
rRNA	Ribosomal RNA
ROV	Remotely Operated Vehicle
RT-PCR	Real Time Polymerase Chain Reaction
SAV	Salmon alphavirus
SDS	Sodium Dodecyl Sulfate
SEM	Scanning Electron Microscopy
SGS	Sediment Grain Size
SNP	Single Nucleotide Polymorphism
SP.	Species singular
SPP.	Species plural
SRS	Salmon Rickettsial Septicaemia
ST	Sequence Type
TE	Typing Efficiency
TEM	Transmission Electron Microscopy
TFZ	Tuna Farming Zone
TVS	Total Volatile Solids
USA	United States of America
USD	United States Dollar
x	Magnification
xg	Centrifugal force

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CHAPTER ONE: GENERAL INTRODUCTION

1.1 GLOBAL AQUACULTURE

Fisheries and aquaculture are the two main areas of aquatic protein production. Fisheries have historically been the main supplier of marine sourced protein with the capture of wild fish. However, as of 2013, 31.4% of fish stocks were estimated to be overfished, 58.1% to be fully fished and only 10.5% considered underfished (1). These numbers indicate that sustainable wild caught fishing is becoming increasingly difficult. While fisheries have been declining, the aquaculture industry has been growing steadily over the past few decades from 7 percent of the combined production in 1974 to 44 percent in 2014 (Figure 1.1) (2).

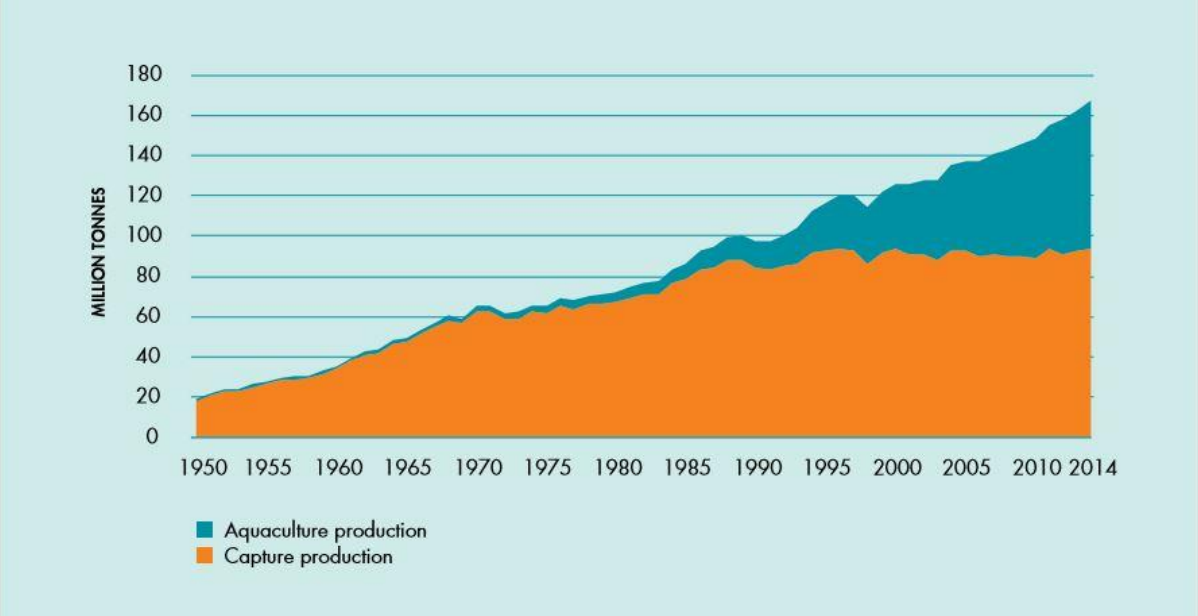

Figure 1.1 Global capture fisheries (orange) and aquaculture (blue) production from 1950 to 2014. (figure from The State of World Fisheries and Aquaculture 2016).

In addition to the increase in the overall production, the number of species cultivated has grown from under twenty to several hundred (1). As the global population continues to grow, aquaculture will become increasingly important to meet the growing demand for sustainable protein (2). Aquaculture is expected to play a large role in contributing to food security and play a key role in adequate nutrition for the expected population of 9.7 billion people by 2050 (1).

By 2014, there were 73.8 million tonnes of fish harvested from aquaculture farms, with an estimated value of 160.2 billion USD. Out of that, 49.8 million tonnes came from finfish production, worth an estimated 99.2 billion USD. Nearly all fish produced were for human consumption (1). Within the finfish section of the aquaculture sector, one of the fastest growing and most profitable areas is production of Atlantic salmon and rainbow trout (1). In 2013, salmonid production was the largest single commodity by value and is expected to increase over time. Within Australia, salmon aquaculture is one of the main primary industries, worth approximately 550 million AUD (3, 4). In addition to Australia, which is a relatively minor salmon producer, salmon aquaculture currently takes place around the world with major producers being Norway, Chile, Canada, Ireland, Iceland, Faeroe Islands (Denmark), Greece, Russia, Spain, Turkey, the United Kingdom, and the United States(1) .

Data suggest that the expansion of aquaculture will only increase with time (5). Unfortunately, with the surge in aquaculture production came the onset of diseases not seen in wild caught fisheries. Infectious marine diseases have a large economic impact and

are one of the major costs associated with aquaculture (6). One such disease that affects fin-fish and particularly, Atlantic salmon, is Amoebic Gill Disease (AGD) (7).

1.2 DISEASE/ PARASITE RISK AND SALMON HEALTH MANAGMENT

Each individual country faces unique challenges in its Salmon farming dictated by specific environmental conditions such as temperature, salinity, current, available location and oxygen saturation (8). Many of these environmental factors contribute to the type of disease causing organisms present and the severity of outbreaks that may occur (8). However, owing to the cosmopolitan nature of many of the causative agents of disease, many salmon-producing countries face the same major health issues (Table 1.1) (8). Aquaculture species can be particularly susceptible to transmittable diseases. New species may be more vulnerable to local infectious agents than wild populations. Increases in stocking density can lead to higher rates of contact, stress and a reduction in water quality leading to optimal conditions for opportunistic infections from bacteria. (6, 9, 10).

Gill diseases in particular can be the source of high mortality in salmon, as gills are in direct contact with the environment and play a significant role in osmoregulation and gas excretion (11, 12). Gill diseases are often of multifactorial aetiology with pathogenicity poorly understood (13) and, in some cases, there can be multiple co-infections with other disease-causing agents and environmental factors such as harmful algal blooms (11).

Table 1.1 Major diseases that affect salmonid aquaculture globally adapted from FAO Fisheries and Aquaculture Department (8).

Disease	Agent	Type	Treatment	Locations Affected
Sea Lice	*Lepeoptheirus salmonis, Caligus elongatus Caligus rogercresseyi*	Ectoparasite	Parasiticide baths or in feed	Norway, Canada, United Kingdom, Faroe Islands, Chile, The United States
Amoebic Gill Disease (AGD)	*Neoparamoeba perurans*	Ectoparasite	Fresh water baths, Hydrogen Peroxide baths	Norway, Chile, United States, United Kingdom, Ireland, Canada, Australia, New Zealand
Infectious Salmon Anaemia (ISA)	*Orthomyxoviridae*	Virus	No Treatment Available	Norway, Canada, Scotland, Faroe Islands, United States, Chile
Infectious Pancreatic Necrosis (IPN)	*Birnavirus*	Virus	Vaccination	The United Kingdom, Chile, Ireland, Scotland, Norway

Infectious Hematopoietic Necrosis (IHN)	*Rhabdovirus* (IHNV)	Virus	Iodine treatment	Austria, Belgium, Canada, China, Croatia, Czech Republic, France, Germany, Iran, Italy, Japan, Korea, the Netherlands, Poland, Russia, Slovenia, Spain, Switzerland, United States (14)
Piscine Reovirus (PRV)/ Heart and Skeletal Muscle Inflammation (HSMI)	Piscine orthoreovirus	Virus	No Treatment Available	Norway, Ireland, Scotland, Canada and Chile
Pancreas Disease (PD)	Salmonid alphavirus (SAV)	Virus	No Treatment Available	Norway, Scotland, Ireland

Furunculosis	*Aeromonas salmonicida*	Bacterium	Antibiotics/ Vaccination	The United States, Canada, The United Kingdom, Scotland, Ireland, Norway, Faroe Islands, Chile
Bacterial Kidney Disease (BKD)	*Renibacterium salmoninarum*	Bacterium	No Available Treatment	Europe (Not Ireland), North and South America
Enteric Red mouth (ERM)	*Yersinia ruckeri*	Bacterium	Antibiotics/ Vaccination in freshwater	Europe, North and South America, Africa, Asia and Australia
Salmon Rickettsial Septicaemia (SRS)	*Piscirickettsia salmonis*	Bacterium	Antibiotics	Chile, Canada, Ireland, Scotland and Norway

1.3 AMOEBIC GILL DISEASE (AGD)

Amoebic gill disease (AGD) is one of the primary diseases facing the salmonid industry, principally Atlantic salmon aquaculture. With increased production comes increased risk of these diseases and outbreaks of AGD have now been reported in every major salmon-producing country with the exception of Iceland (15, 16). In addition to Atlantic Salmon, AGD has also been reported in blue warehou (*Seriolella brama*) in Australia (17); farmed brown trout (*Salmo trutta*) in France (18, 19); farmed ayu (*Plecoglossus altivelis*) in Japan (20); farmed olive flounder (*Paralichthys olivaceus*) in Korea (21); farmed sea bass (*Dicentrarchus labrax*) in the Mediterranean (22); farmed Chinook salmon (*Oncorhynchus tshawytscha*) in New Zealand (23, 24); Ballan wrasse (*Labrus bergylta*) and lumpsuckers (*Cyclopterus lumpus*) in Norway and Scotland (25); horse mackerel (*Trachurus trachurus*) in Scotland (26) and farmed turbot (*Scophthalmus maximus*) in South Africa and Spain (22, 27-29) (Figure 1.2). Interestingly, there have been no reports of AGD in wild salmon populations (30).

Amoebic gill disease presents as visible, multifocal white lesions on the gills caused by the host reaction to the attachment of amoeba (31). Hyperplasia of the epithelial and mucosal cells occurs along with the fusing of lamella (31). The presence of white patches or lesions is used by the salmon industry as an indicator of the severity of the disease, which can range from one or two small patches near the gill arch to multiple large patches covering a significant portion of each gill (32-34). The fusing of lamellae reduces the functional surface area of the gills causing a potential decrease of gas exchange; this, coupled with excess mucus production leads to prolonged respiratory distress and eventually suffocation (35). The respiratory distress contributes to loss of appetite and

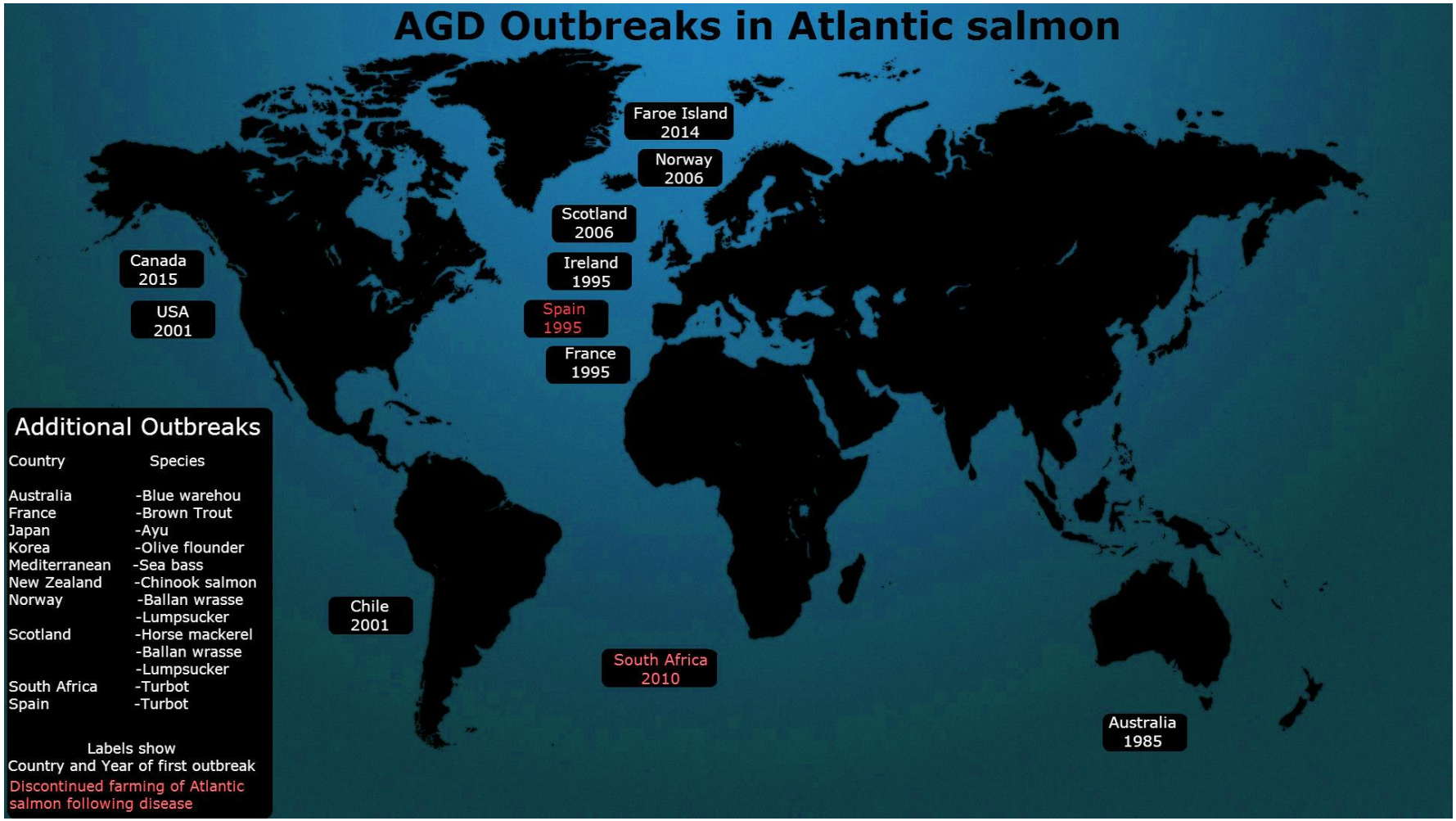

Figure 1.2 AGD outbreaks in Atlantic salmon along with the year of the first recorded outbreak. Red lettering indicates countries where farming was discontinued, insert shows additional species with AGD outbreaks by country. (Figure from Johnson-Mackinnon et al. 2016).

Table 1.2 AGD Impact and Current Situation for Atlantic salmon aquaculture by country, based on Oldham et al. 2016.

Country	AGD Impact	Current Situation	Reference
Norway	Up to 82% mortality	Recurring problem	Steinum et al. 2008 (36), Rodger 2014 (19), Powell et al. 2015 (37)
Scotland	Up to 70% mortality	Recurring problem	Young et al 2008a (24), Rodger 2014 (19)
Chile	Up to 53.8% mortality	Recurring problem	Munday et al. 2001 (18), Nowak et al. 2002 (38), Bustos et al. 2011 (39), Rozas et al. 2012 (40)
Australia – Tasmania	Up to 50% mortality	Recurring problem	Munday 1986 (41), Douglas-Helders et al. 2001 (42, 43), Young et al. 2007 (44)
USA – Washington	Up to 21% mortality	Recurring Problem	Douglas-Helders et al. 2001 (43, 44), Young et al. 2008a, b (24, 45), Nowak et al. 2010 (46)
South Africa	Approx. 5% annual mortality	Project discontinued	Mouton et al. 2014 (27)
Spain	Major	Farming Discontinued	Rodger and McArdle 1996 (47), Munday et al. 2001 (18)

France	Minor	Sporadic	Findlay et al. 1995 (23), Rodger and McArdle 1996 (47), Munday et al. 2001 (18)
Canada – British Columbia	Minor	Sporadic	ICES 2015 (48)
Faroe Islands	No mortalities	First recorded 2014	Oldham et al. 2016 (15)

reduced growth over short time periods or eventual mortality long term if the disease is severe and left untreated (7, 49).

The severity and recurrence of AGD in farmed Atlantic salmon varies greatly across countries (15) (Table 1.2). In some countries such as Norway, outbreaks of AGD are an annual occurrence and have been reported to cause up to 82% mortality in salmon smolts when left untreated (36). In Tasmania, where re-occurrence is a common issue, AGD can cause up to 50% mortality and require up to 15 treatments a year where as in other countries such as the Faroe Islands no mortalities have been reported (15, 19, 50). There can be high costs associated with AGD outbreaks depending on the type, method and level of treatment and their respective labour costs along with reduced growth and loss of product (11, 51). Though there has been significant research into treatments for AGD, there are only two main methods of treatment available to commercial farms: fresh water bathing and hydrogen peroxide bathing (15, 16). Fresh water bathing has been the preferred method of treatment since the emergence of AGD(52) and has been shown to be effective with an 86 +/- 9.1% reduction in amoeba (53). The method works by changing the osmolarity causing a decrease in gill mucus and the detachment of cells. The reaction causes the amoebic cells to oxidise and damages the cell membrane causing the eventual bursting of amoeba cells (54).

1.4 THE CAUSATIVE AGENT OF AGD

It is now known that the causative agent of AGD is *Neoparamoeba perurans* which was identified in 2007 as an aetiological agent and confirmed via Koch's postulates in 2012

(41, 44, 55). The genus *Paramoeba* was first described in 1896 by Schaudinn who isolated the amoeba from a marine aquarium in Germany and named it *Paramoeba eilhardi* (56). As this was the first species named within the genus *Paramoeba, P. eilhardi* became the type species for the genus. Following the identification of *Paramoeba,* and prior to the wide spread use of 18s rRNA gene sequencing, a sister genus, *Neoparamoeba,* was described based surface morphology separating *Paramoeba* into two genera : *Paramoeba* and *Neoparamoeba* (57). Amoebae of the genus *Neoparamoeba,* are distinguished from the closely related sister genus *Paramoeba* by the absence of surface structures, such as scales (57). *Neoparamoeba* are small (typically 10 – 30µm in length) lobose amoeba belonging to the family Vexilliferidae (44, 57). These amoebae have variable pseudopodia forming large broad edge pseudopodia in their attached motile form and long narrow pseudopodia in their suspended form (57). *Neoparamoeba/paramoeba* are facultative parasites, not requiring a host to complete their lifecycle (58). They are considered to be free-living but can become opportunistically parasitic (58). In many instances, co-isolation of amoebae from gills of infected fish species resulted in the identification of multiple species of *Paramoeba* and *Neoparamoeba* (Table 1.3) (59). As the co-isolation would suggest, other members of the *Neoparamoeba/paramoeba* genera besides *N. perurans* are known to be parasitic (Table 1.3). Notably *Neoparamoeba invadens* in green sea urchins (*Strongylocentrotus droebachiensis*) and *Neoparamoeba pemaquidensis* in lobster (60-62). Paramoebiasis can cause mass mortalities in the affected species and can cause significant impact economically for industry (58).

Since molecular studies based on 18S rRNA gene sequences have become more wide spread there is evidence to suggest that *Paramoeba* and *Neoparamoeba* should be

synonymous and revert to a single genus, *Paramoeba,* however before this can be fully adopted more sequencing of *Paramoeba* and *Neoparamoeba* species samples should occur (57, 62). In order to fully understand the phylogenetic relationships between thesis species, more sampling of underrepresented species (*P. eilhardi, P. atlantica*, *N. aestuarina* and *N. longipodia*) is needed. For the purpose of this thesis, the current taxonomic consensus has been adopted, and *Neoparamoeba perurans* will be used when referring to the parasite.

The most distinguishing feature of *Neoparamoeba* and *Paramoeba* is the presence of a peri-nuclear body, termed a parasome. Most species have retained one parasome, though multiple parasomes per cell have been observed (63). When coevolutionary studies have been conducted on *Neoparamoeba* and *Paramoeba* species, parasome phylogenies appeared to mirror amoebae phylogenies indicating that the parasome is passed on from mother to daughter cells, suggesting a strong hereditary component (62, 64). The parasome itself contains the reduced form of a eukaryotic cell from an evolutionary endosymbiosis event of an kinetoplastid protozoan (56, 65). The parasome contains a disk-shaped mass of DNA, or 'kinetoplast', within the mitochondria. A study on *N. pemaquidensis,* discovered that the parasome and amoeba are inter-related and share cellular and metabolic functions (66). How this connection between the parasome and amoeba nucleus affects the pathogenicity of amoebae remains unknown (66). However, considering the parasitic nature of some kinetoplastid members (i.e. *Trypanosoma*) the parasome could be involved in a yet unknown way (66).

Analysis of the 18S rRNA gene sequence is a commonly used technique in eukaryotic phylogenetic studies, in part because the large number of copies allows for high levels of

sensitivity (59, 67, 68). To be reliable for determining clades in phylogenetic studies, a gene must be robust against mutation and cannot have too many polymorphic sites. Specifically, the 18S rRNA gene is the standard for determining interspecies phylogenetic relationships within *Neoparamoeba* and *Paramoeba,* but the gene lacks the polymorphic variation to differentiate at the intra-species level between *N. perurans* samples (45). The internal transcribed spacer (ITS) genes (ITS1, 5.8s and ITS2) have also been examined in relation to *Neoparamoeba but* appeared too polymorphic to characterise either *N. pemaquidensis* or *N. perurans* samples (69). This is because of apparent intra-genomic sequence heterogeneity in the ITS regions of clones of *N. pemaquidensis*, one of the closely related sister species *N. perurans* (69).

Table 1.3 Known *Neoparamoeba* and *Paramoeba* species including the name, species and/or environment association, disease association, location and source.

Species	Species/ Environment Found	Disease Associated	Location	Source
Paramoeba eilhardi	Marine environment	No	North Atlantic Oceans	Schaudinn 1896 (56)
Paramoeba perniciosa	Blue Crab (*Callinectes sapidus*)	Yes	Eastern seaboard, USA	Sprague et al. 1969 (70), Johnson 1977 (71), Messick 2002 (72)
Paramoeba schaudinni	Marine environment	No	Brazil	De Faria et al. 1920 (73)
	Bottom Sediments	No	Gulf of Mexico	Sawyer 1980 (74)
Paramoeba atlantica	Deep-sea sediments	No	Eastern Atlantic Ocean	Kudryavtsev et al. 2011 (63)
Neoparamoeba branchiphila	Atlantic Salmon (*Salmo salar*)	No	Tasmania, Australia	Dyková et al. 2005 (59)
	Blue Crab (*C. sapidus*)	No	Gulf of Mexico, USA	Dyková et al. 2007 (75)

	Sea Urchin (*Diadema* aff. a*ntillarum*)	Yes	Canary Islands, Spain	Dyková et al. 2011 (76)
	Sea Urchin (*Heliocidaris erythrogramma*)	No	Tasmania, Australia	Dyková et al. 2007 (75)
	Sea Urchin (*Paracentrotus lividus*)	No	Cretan Sea, Greece	Dyková et al. 2007 (75)
	Southern Bluefin Tuna (*Thunnus maccoyii*)	No	Port Lincoln, Australia	Dyková et al. 2007 (75)
Neoparamoeba pemaquidensis	American Lobster (*Homarus americanus*)	Yes	Long Island Sound, USA	Mullen et al. 2004 (60)
	Atlantic salmon (*S. salar*)	No	Tasmania, Australia; Ireland	Roubal et al. 1989 (77), Wong et al. 2004 (67), Dyková et al. 2007 (75), Douglas-Helders et al. 2002 (78)
	Coho salmon (*Oncorhynchus kisutch*)	No	Washington State and California, USA	Wong et al. 2004 (67), Kent et al. 1988 (79)
	Sea Urchin (*S. drobachiensis*)	No	Gulf of Maine, USA	Caraguel et al. 2007 (80)

	Southern Bluefin Tuna (*T. maccoyii*)	No	Port Lincoln, Australia	Dyková et al. 2007 (75)
	Turbot (*Scophthalmus maximus*)	Yes	NW Spain	Dyková et al. 1998 (29), Fiala & Dyková 2003 (81)
	Benthic Sediments	No	Tasmania, Australia	Crosbie et al. 2003, 2005 (82, 83)
Neoparamoeba perurans	Atlantic salmon (*S. salar*)	Yes	Chile, Ireland, Norway, Scotland, USA, Australia	Bustos et al. 2011 (39), Young et al. 2008 (84), Steinum et al. 2008 (36), Young et al. 2007 (44), Bridle et al. 2010 (68), Crosbie et al. 2012 (55), Nowak et al. 2010 (46)
	Ayu (*Plecoglossus altivelis*)	Yes	Japan	Crosbie et al. 2010b (20)
	Chinook Salmon (*Oncorynchus tshawytscha*)	Yes	New Zealand	Young et al. 2008 (84)
	Rainbow Trout (*Oncorynchus mykiss*)	Yes	Tasmania, Australia	Young et al. 2008 (84)
	Turbot *(S. maximus*)	Yes	NW Spain	Young et al. 2008 (84)

Neoparamoeba invadens	Sea Urchin (*Stongylocentrots cdroebachiensis*)	Yes	Nova Scotia, Canada	Jones 1985 (61), Jones & Scheibling 1985 (85), Jones et al. 1985 (86), Jellett & Scheibling 1988b (87), Feehan et al. 2013 (62)
Neoparamoeba aestuarina	Marine environment	No	North Atlantic Ocean/Sea of Japan	Page 1970 (88), Volkova and Kudryavstev 2017 (89)
Neoparamoeba longipodia	Deep-sea sediments	No	Western Atlantic Ocean	Volkova and Kudryavstev 2017 (89)

1.5 DETECTION OF *NEOPARAMOEBA AND PARAMOEBA*

MICROSCOPIC IDENTIFICATIONS

One of the early and primary ways of discerning if an amoeba was a member of the *Neoparamoeba* and *Paramoeba* genera was the use of microscopy. Prior to the initial outbreaks of amoebic gill disease in finfish, six members of *Neoparamoeba* and *Paramoeba* genera had already been isolated and named (79). They were *P. aestuarina* (88), *P. eilhardi* (56), *P. schaudinni* (73), *P. perniciosa* (70), *P. invadens* (61) and *P. pemaquidensis* (88). Amoebae are attributed to *Paramoeba* primarily on the basis of the presence of the parasome near the nucleus under DIC or Light microscopy (7, 41). Surface ultrastructures are often difficult to see using light microscopy and determination of surface scales and parasome is done using Scanning Electron Microscopy (SEM) or Transmission Electron Microscopy (TEM) (75, 79). Individual species can be differentiated on the basis of pseudopodial structure, number of parasomes present, the presence or absence of surface filaments and the length to width ratio observed (79). There are, however, issues with basing an identification purely on microscopic measurements. There are often morphological similarities between different species of amoeba and marked differences in size within the same species as described by Dyková et al. 2005 (22, 59). Light microscopy it still useful in determining if an amoeba contains a parasome and is thus a member of *Neoparamoeba* and *Paramoeba.* It is not however, reliable in identifying the species (59).

DETECTION ON GILLS

Histology is one of the primary methods of diagnosis of AGD, particularly in instances when other factors are present that could cause gross gill pathology such as water bourn irritants or environmental changes (11, 31, 90). In addition to the diagnosis, histology can also be used in gill scoring for determination of overall gill health (90). However, it is impossible to determine the amoeba species using histology as amoebae have no distinguishing morphological features (45).

Quick Dip® and Immunofluorescence antibody test (IFAT) which are haematology stains, can be used as identifiers for AGD (91). In a study on gill smear staining, IFAT and Quick Dip® were shown to be statistically indistinguishable for the identification of amoeba cells (91). IFAT uses polyclonal antibodies, generally either rabbit or goat, raised against *Neoparamoeba* species mainly *N. pemaquidensis* and thus has a greater specificity than Quick Dip® (24, 38, 43, 91). However, the antibodies used in the IFAT assay have also been shown to be non-specific in some instances and early confirmation of AGD causing amoebae relying on IFAT alone could be any of the known gill derived *Neoparamoeba* species: *N. branchiphila, N. pemaquidensis* or *N. perurans.*

MOLECULAR BIOLOGICAL DETECTION TECHNIQUES

The most definitive methods for determining the species present on the gills is the application of molecular tools such as polymerase chain reaction (PCR) and in situ hybridization (ISH) (11, 34, 67, 75, 92-95). In particular, PCR detection which has become routinely used for diagnostic and phylogenetic studies (11). Species-specific primers are generally created to amplify an approximate 600 bp region of the 18S rRNA gene. The 18S

rRNA gene has a high copy number (2880 per amoeba) and thus allows for high levels of sensitivity (68). However, there were and may still be some issues present in sequencing analysis. For instance, prior to 2007, *N. pemaquidensis* was presumed to be the causative agent therefore studies focused on confirming its presence on infected gills using species-specific primers (67). These primers however, were shown to be not specific for amplification of *N. pemaquidensis* DNA as there were instances of primers also amplifying *N. branchiphila* DNA (83). Consequently, historical references can be used to confirm the presence of *Neoparamoeba* spp., but the identification of the specific species is not necessarily accurate.

There can also be issues associated with the methods behind the PCR conducted such as the type of reaction and reagents used (11). Differences between PCR, which requires enough amplified DNA to be visible under UV light on a stained gel, and quantitative (q) PCR, which detects the DNA in real-time during the amplification process will have different levels of sensitivity in detection (68). The reagents used for qPCR can also have an impact because differences between SYBR® Green and TaqMan® in *N. perurans* detection assays have been reported (11, 68, 96).

These factors can influence the sensitivity of an assay which is of particular importance in environmental surveys when low numbers of amoebae are suspected (68, 96). Despite methodological differences, fish have tested positive (at least the presence of *N. perurans* on the gills) using PCR despite displaying no gross pathology and being negative under histological examination. This would indicate that PCR is more sensitive than traditional diagnostic tools (Histology, IFAT etc.) and thus should be the standard for positive *N. perurans* detection (24, 45, 97). However, since the sensitivity of PCR,

particularly qPCR, is so high there is the risk that positive results could be due to few cells coincidently in the vicinity or accidently transferred during initial fish handling (68).

1.6 MOLECULAR TECHNIQUES FOR INTRA AND INTER SPECIES DETERMINATION

Recognizing genetic divergence within species, even when the species does not display reproductive or morphological divergence, allows for better understanding of speciation and biodiversity (98). To that end, there are several molecular typing methods currently in use, the main four being: Pulsed-Field Gel Electrophoresis (PFGE), Restriction Length Polymorphisms (RFLP), Multilocus Sequence Typing (MLST) and Random amplified polymorphic DNA (RAPD) (99). Of those four methods, two have previously been used on *Neoparamoeba* species. Pulsed-field gel electrophoresis was used to better understand and characterize the genome of the parasome and nucleus of *N. pemaquidensis* (100). The method involves periodically changing the electrical field during gel electrophoresis in order to separate large (over 1000 kb) DNA molecules that would otherwise fail to migrate properly (99, 100). PFGE was useful in the context of understanding the structure of the two genomes, but the PFGE gel produced a large smear rather than a clear banding pattern (100). For this reason, PFGE is not a good choice for the comparison of samples.

RFLP was applied to *N. pemaquidensis* and used restriction enzymes to recognize short sections of genomic DNA sequences and fragment the genome through restriction digest (80, 99). The ITS region of the genomes of both parasome and amoeba was analysed this way in *N. pemaquidensis* (80). The nuclear ITS region had too much microheterogeneity to be a reliable marker, but when combined with additional markers for the parasome, the

analysis was able to differentiate between samples from an outbreak in Washington (69, 80). Since this microheterogeneity is present in *N. pemaquidensis,* and length heterogeneity of the ITS region has been observed in *N. invadens* (62), it is likely that it also exists in some form in *N. perurans* requiring the same type of combined analysis which is not ideal when comparing a large number of samples. The last two typing methods, MLST and RAPD are less time consuming and are therefore good candidates for *N. perurans.*

Multilocus Sequence Typing (MLST) is a PCR-based technique that compares gene sequences from several loci, where differentiation of types is based on the number of nucleotide polymorphisms per allele per gene (101). MLST was first described in a study on the bacterium *Neisseria meningitis* (101). The technique has since been used to reveal genetic diversity in other prokaryotic populations including important fish pathogens (102-108). MLST analysis have been applied to *Yersinia ruckeri*, the aetiological agent of enteric redmouth disease (ERM) in salmonid fish (102). Typing was able to determine the relationship between sequence types and host specificity as well as link sequence types to virulence in *Y. ruckeri* determining that ERM was originally a geographically isolated disease that spread rapidly to a large area (102). MLST has also been applied to a growing variety of eukaryotic organisms as a typing method, including other parasitic organisms including kinetoplastida such as *Trypanosoma cruzi* and amoebae including *Entamoeba histolytica* and *Acanthamoeba* spp. (109-113).

The final typing method, Random Amplified Polymorphic DNA (RAPD), was first developed by Williams (114) as an alternative to Random Fragment Length Polymorphisms (RFLP) for the creation of genetic maps. RAPD assays rely on a number of ten base pair primers that lack palindromic sequences and have a high CG content (115). As there is a high

probability that most genomes contain several small inverted repeats close together, when amplified through PCR, the short RAPD primers bind to these small inverted repeats within the genome and amplify the intervening DNA segments which produces unique gel profiles (116). RAPD has gained popularity due to several factors; a) there is no need for prior genomic sequencing information which makes it very useful in non-model organisms, b) relative to other molecular methods such as MLST which requires sequencing RAPD is low cost, and c) RAPD is a relatively quick method for determining genetic differences between and within species (115).

Table 1.4 General benefits and disadvantages to four main typing methods consided for *N. perurans*

Typing Method	Benefits	Disadvantages	Reference
Pulse-field Gel Electrophoresis (PFGE)	No need for prior sequence information i.e. primer design; Useful for non-model organisms; Low relative cost Quick results – no need for sequencing; Discriminatory power in subtyping	Patterns tend to be smeared and hard to compare/ reproduce Pattern can be changed by electrolysis setting or gel composition	Stepan et al. 2011 (118) Hammerum et al. 2015 (119)
Random Fragment Length Polymorphism (RFLP)	Consistent patters – easily reproducible; No need for prior genomic knowledge;	Cannot separate between target and contamination;	Harun et al. 2009 (120) Bart-Delabesse et al. 2001 (121) Powell et al. 1996 (122)

	Useful on non-model organisms; Low relative cost	Length heterogeneity within genes can change banding pattern affecting reproducibility	
Random Amplified Polymorphic DNA (RAPD)	No need for prior genomic knowledge; Useful on non-model organisms; Low relative cost Quick results – no need for sequencing	Requires strict quality control; Cannot separate between target and contamination; Absence of standardized methodologies; Issues with reproducibility	Harun et al. 2009 (120) Bart-Delabesse et al. 2001 (121) Powell et al. 1996 (122)
Multilocus Sequence Typing (MLST)	Sequencing allows for target organism certainty; Easy to compare across large numbers of samples	High strain discrimination requires correct gene selection; Accurate sequence determination is crucial; Higher	Harun et al. 2009 (120) Stepan et al. 2011 (118) Hammerum et al. 2015 (119)

		cost - Requires sequencing of results for comparison;

1.7 DETECTION OF *Neoparamoeba perurans* IN THE ENVIRONMENT

Amoebae of the genera *Neoparamoeba* and *Paramoeba* are considered marine or estuarine organisms (57). Species within both genera have been isolated from marine organisms, water columns and marine sediments (Table 1.3). Except for heavy infections of marine organisms, detections have been in small numbers in all sampled substrates. Since *N. perurans* is a facultative parasite it does not have intermediate hosts which are used as reservoirs. It is unknown if *Neoparamoeba* have source populations in the environment that help contribute to the high rates of infection seen on salmon farms.

Since 1998, there have been studies into the presence of the causative agent of AGD in non- salmonid species and associated environmental locations (15). However, since the emergence of the disease in the 1980s and until the causative agent was confirmed in 2012, AGD was attributed to known *Neoparamoeba* species (15, 41, 55). Initially, and for the majority of the time period, *N. pemaquidensis* was considered to be the causative agent and thus many of the environmental studies done in attempts to identify the pathogen in the environment focused either on *N. pemaquidensis* or did not go beyond the genus level (78, 82, 83, 117). Regardless of the lack of specificity to *N. perurans*, there have been studies that show *Neoparamoeba* and *Paramoeba* species have been found together in marine environments and as co-infection agents on the gills of salmon (81). Therefore, these studies still have relevance in determining the presence of *N. perurans.* The focus of previous studies can be generalized into four sections: water, biofouling, wild fish species and sediment (15, 118).

The method of both sampling and confirming the presence of *Neoparamoeba* species in water column has varied (see Table 1.5) which makes direct comparisons between studies difficult. In addition, the majority of these studies were in Tasmania, with only one study done in another geographic location, Norway (68, 117-119). The first study within Tasmania was conducted by Tan et al. (2002) and employed a variety of techniques to determine the most effective (Table 1.5) (117). The water samples were taken at two time points, May and June, and from within both the seacages and the surrounding water although a thorough description of sampling location and depth was not included (117). All techniques produced positive results for *N. pemaquidensis.* No quantification of amoeba number was done in this study, rather the success rate of each sampling was reported as a percentage of IFATs that were positive, none of the methods reported a positive percentage above 33% (117). In 2003 another study was conducted in Tasmania, again focusing on *N. pemaquidensis* (120). Samples were taken from a variety of location around Tasmania as well as a variety of depths, all were processed using the Immuno-dot blot test and 9 samples were confirmed via PCR (120). All sites had samples that returned positive results, as with the previous study no quantification of amoebae number was done (120). Subsequent studies in Tasmania and Norway focused on *N. perurans* (68, 118, 119) because they occurred after this species was shown to be the causative agent of AGD. One study used samples collected by divers and the remaining two used samples collected by a Niskin bottle (68, 118, 119). All three studies used filtration, DNA extraction and PCR to determine the presence of amoebae though the exact methods varied. Regardless, *N. perurans* was identified in all locations though only in low numbers in water samples taken near or within seacages in Australia and Norway where AGD outbreaks were ongoing (15, 118).

Biofouling is a potential reservoir for *N. perurans* due in part to the diverse assortment of organisms present that could potentially carry the pathogen. For instance bivalve molluscs have been shown to bio-accumulate infectious hematopoietic necrosis virus (IHNV) through their filter feeding processes (121). In addition, net and pen surfaces are often a source of microfouling – a film of micro-organisms including bacteria- which would provide ample food source for the amoebae (117). When biofouling was surveyed in Tasmania during AGD-related studies all the biofouling organisms tested positive for *N. pemaquidensis* (Table 1.6) (122). However, when biofouling organisms from Western North America were tested for *N. perurans,* including in regions known to have AGD outbreaks, none were positive, possibly due to lack of sensitivity of the method used (46). The most recent survey of biofouling organisms done in Norway using qPCR did find *N. perurans* DNA in 20 of the 874 organisms sampled (from Bryozoa, Chordata, Mollusca and Cnidaria) (Table 1.6). The samples that were positive came from one time point sampled for one farm that was, similar to Tan et al. (2002), undergoing an AGD outbreak at the time of sampling (118).

Table 1.5 List of all surveys conducted of the water column for *Neoparamoeba* sp. thought to be associated with amoebic gill disease. Methods varied between studies however all studies were positive for amoebae and PCR confirmed at the end. Only one study was prior to 2008 when the creation of a *N. perurans* specific PCR was created. * indicates instances where the determined species may have been misidentified.

Specificity	Location	Sampling method	Source
*N. pemaquidensis**	Tasmania	1) Water (50 mL) filtered/ filter placed on MYS for grow out 2) Water 3-5 L filtered/ 1-2 mL of retained/ liquid inoculated onto MYS for grow out 3) filter from method 2 inoculated onto MYS for grow out 4) 1-2 mL sample from plankton tows onto MYS for grow out All) Growth smeared on glass slide/ IFAT tested/ PCR confirmed	Tan et. al. 2002 (117)
*N. pemaquidensis**	Tasmania	Diver collection/ Immuno-dot blot/ PCR confirmed	Douglas-Helders et al. 2003 (120)
N. perurans	Tasmania	Diver collection /Filtration/ kit DNA extraction/ RT-PCR	Bridle et al. 2010 (68)

N. perurans	Tasmania	Niskin bottle collection/ Filtration/ Isopropanol extraction/ RT-PCR	Wright et. al. 2015 (119)
N. perurans	Norway	Niskin bottle collection/ Filtration /QIAzol Incubation/ Scalpel removal of biological matter on filter/ liquid and small section of filter analysed/ RT-PCR	Hellebø et al. 2017 (118)
N. perurans	Tasmania	Niskin bottle collection/ Filtration/ Isopropanol extraction/ RT-PCR	Wright et al. 2017 (123)

Analogous to the studies on biofouling, there have been large surveys of wild fish species commonly found in association with salmon aquaculture or, species that are known to be cleaner fish species commonly associated with salmon seacages (15). Prior to the most recent study in 2017, only two in over five thousand fish surveyed were positive for the presence of *N. perurans* (Table 1.7) (17, 26). Fish associated with seacages that did test positive were cleaner fish species or those found in or near seacages where salmon were undergoing an AGD outbreak (17, 25, 26, 79, 118). Comparably, the most recent survey done in Norway found 35 fish that tested positive for *N. perurans* (Table 1.7) (118). Though a higher proportion of fish tested positive for AGD in this study, the results came from a farm undergoing a heavy AGD outbreak as with other reported positives (118). Interestingly, the same farm at the same time point had positive *N. perurans* association with biofouling organisms as well. This indicates that when non-cultured cage associated organisms are positive for *N. perurans* it is in a strong association with active AGD outbreaks (118).

Table 1.6 List of all reported surveys conducted on biofouling and biofouling-related species for *Neoparamoeba* sp. though to be associated with amoebic gill disease including details on the specific species surveys. * indicates instances where the determined species may have been misidentified.

Study Specificity	Location	Species surveyed	Confirmation Method	Confirmed	Source
*N. pemaquidensis**	Tasmania, AUS	Skeleton shrimp, amphipod, Solitary ascidians (*Mogula ficus, Ciona intesinalis)*, Colonial ascidian, hydroid (*Obelia australis*), bryozoan (*Scrupocellaria bertholettii*), blue-lip mussel (*Mytilus edulis*), shrimp (*Macrobrachium* sp.), crab, marine worm (*Eunice* sp.)	IFAT	Yes (all)	Tan et al. 2002 (117)
N. perurans	Vancouver Island, CAN/	22 organisms sampled: Skeleton shrimp (*Caprella* sp.), Mussels (*Mytilus* sp.), anemones, urchin, sponge, Macroalgae, periphyton	PCR	No (all)	Nowak et. al 2010 (46)

	Puget Sound, USA				
N. perurans	Norway	874 organisms sampled: Beach hoppers (*Amphipoda*), edible crab (*Cancer pagurus*), skeleton shrimp, green crab (*carcinus maenas*), isopod (*Idotea* spp.), squat lobster (*Munida* sp), hermit crab, barnacle (*semibalanus balanoides)*, sea spider, Bryozoa, Chordata, Dead man's fingers (*Asterias rubens*), Sea anemones, knotted thread hydroid, *Tubularia* spp., jelly fish, Comb jelly, Sea star, common starfish (*Asterias rubens*), snowflake star (*Crossaster*	Real Time PCR	Yes (partial)	Hellebø et al. 2017 (118)

papposus), feather stars, sea urchin, brittle star, Saddle oyster (*Anomia ephippium*), wrinkled rockborer (*Hiatella arctica*), blue mussel, scallop (*Pecten* spp.), topshell (*Gibbula* spp.), periwinkle (*Littorina* sp.), nassa mud snails (*Nassarius* spp.), dog winkles (*Nucella* spp.), sea slugs, limpet (*Patella* spp.), cowry (*Trivia* spp.), valve snail (*Valvata* spp.), ribbon worm and sponges

Table 1.7 List of all reported *Neoparamoeba* sp. surveys conducted of wild fish species associated with or found near salmon seacages. Details on the target species termed "specificity", location of surveys, number and species surveyed, method used for analysis and the publication have been included. * indicates where *N. pemaquidensis* was the target amoebae as it was believed to be the aetiological agent at the time prior to the confirmation of *N. perurans* as the causative agent of AGD. The detection methods were not species specific and would detect *N. perurans* as well.

Specificity	Location	Species	Confirmation Method	Source
Neoparamoeba sp.	British Columbia	2969 wild fish were surveyed, 0 positive: The majority were Chinook salmon (*Oncorhynchus tshawytscha*), Chum salmon (*Oncorhynchus keta*), Coho salmon (*Oncorhynchus kisutch*), Sockeye salmon (*Oncorhynchus nerka*), Pink salmon (*Oncorhynchus gorbuscha*)	Histology	Kent et al. 1998 (124)
*N. pemaquidensis**	Tasmania	325 wild fish surveyed, 0 positive: 12 species collected only the most common named - Jack mackerel	Histology/ IFAT	Douglas-Helders et al. 2002 (78)

		(*Trachurus declivis*), Sand flathead (*Platycephalus bassensis*) Red cod (*Pseudophycis bachus*)		
Neoparamoeba sp.	Tasmania	1 wild fish opportunistically caught, *N. perurans positive*: Blue warehou (*Seriolella brama*)	Histology/ IFAT	Adams et al. 2008 (17)
N. perurans	Norway	Cultured Ballan wrasse (*Labrus bergylta*) *N. perurans* positive	Histology/ qPCR	Karlsbakk et. al. 2013 (25)
N. perurans	Scotland	2348 wild fish surveyed, 1 *N. perurans positive*: Horse Mackerel (*Trachurus trachurus*)	qPCR	Stagg et al. 2015 (26)
N. perurans	British Columbia	86 adult Chinook Salmon (*Oncorhynchus tshawytscha*) surveyed, 0 positive.	qRT-PCR	Bass et al. 2017 (125)
N. perurans	Norway	197 wild fish surveyed, 35 *N. perurans positive*: Lumpsucker (*Cyclopterus lumpus*), Atlantic cod (*Gadus morhua*), Saithe (*Pollachius virens*), Sculpin	qPCR	Hellebø et al. 2017 (118)

(*Myoxocephalus scorpius*), Cod (*Gadhus morhua*),

Tadpole fish (*Raniceps raninus*), Goldsinny wrasse

(*Ctenolabrus rupestris*), Ballan wrasse (*Labrus bergylta*),

Cuckoo wrasse (*Labrus bimaculatus*), Corkwing wrasse

(*Symphodus melops*), Rock gunnel (*Phois gunnellus*)

The fourth potential reservoir, the sediment, has historically been the most challenging and least well surveyed. The first survey of sediment in 2003 focused on *Neoparamoeba pemaquidensis,* and a subsequent one in 2005 on *Neoparamoeba* sp. as co-isolation of amoeba species from AGD infected gills was reported (82, 83). Both studies were conducted on sediments from both salmon farming and non-salmon farming sites around the coast of Tasmania (Table 1.8) (82, 83). Though these studies both returned positive results they relied on a culture enrichment (malt yeast agar) followed by identification with IFAT and PCR once amoebae grew and migrated from the sediment inoculation point on the agar (82, 83). The next survey conducted was along the Western coast of North America from naive and in farm associated sites, used small samples preserved in ethanol after sampling and prior to DNA extraction (Table 1.8) (60). Following that study another was conducted in Norway from farm sites in 2017 using a $2mm^3$ sample preserved prior to RNA extraction (Table 1.8) (118). In both the North American and the Norwegian studies, there were no positive identifications of *N. perurans* DNA (46, 118).

Table 1.8 List of all surveys conducted for the detection of *Neoparamoeba* sp. in sediments including details on the methods for comparison. * indicates instances where the determined species may have been misidentified.

Specificity	Location	Method	Confirmed	Source
*N. pemaquidensis**	Tasmania	Diver collection 3-5g of sediment smeared onto mya plates/ IFAT/ DIC with DAPI / PCR Sample positive when all three methods positive	Yes	Crosbie et al. 2003 (82)
Neoparamoeba sp.	Tasmania	Van Veen grab sampler 3-5g spread onto mya plates / IFAT/ PCR (*N pemaquidensis and N. branchiphila)*	Yes	Crosbie et al. 2005 (83)
N. perurans	Western North America	Diver collection Fixed in 10 mL 95% ethanol/ Kit DNA extraction (1mL)/ PCR	No	Nowak et al. 2010 (46)

N. perurans	Norway	Collected with a van Veen grab (250cm^2) 2mm^3 was incubated in QIAzol/ RNA purification/ qPCR	No	Hellebø et al. 2017 (118)

Despite the positive identifications of *N. perurans* in the water column, biofouling and related fish species, all these sources are considered unlikely reservoirs since positive identifications of *N. perurans* only occurred when gross signs of AGD were visible in fish and at very low levels when detected (55, 56, 58). Sediment, at present, is also considered an unlikely reservoir of *N. perurans* as there have been no identification of *N. perurans* specifically (66). However, there has been identification of *Neoparamoeba* spp. from sediments around Tasmania including at locations where no farming has occurred (82, 83).

1.8 OBJECTIVES

Several gaps have been identified in our knowledge of AGD, particularly related to *N. perurans* ,the parasite responsible for this disease (15, 16). The overall goal of this thesis was to investigate the relationship between *N. perurans* samples and the environment. In order to investigate this, molecular methods were employed to study:

1) relationships between geographically diverse samples using multilocus sequence typing and Random Amplified Polymorphic DNA

2) environmental presence of *N. perurans* through sediment handling methodologies and DNA extraction assays to facilitate molecular detection.

CHAPTER TWO: MULTILOCUS SEQUENCE TYPING (MLST) OF GEOGRAPHIC SAMPLES OF *Neoparamoeba perurans*

2.1 INTRODUCTION

Species and sub-species typing has been used in an assortment of species ranging from bacteria and viruses through to fungi, microbial eukaryotes and vertebrates (109, 110, 126-130). MLST was initially developed to solve two types of issues: typing isolates from localized disease outbreaks to determine how many types of strains were responsible and how localized disease strains from one geographic area related on a global scale (101). Typing can be particularly important in parasitic or pathogenic species as changes in environment or genetic divergence can lead to new host specificity or target location within a host (131). Identifying these phenomena early can help identify new potential threats before they become widespread or epidemic. This can be particularly important for pathogenic species that impact human health or commercially important species.

Two examples of typing applied to human disease associated species are the amoebic genera *Acanthamoeba* and *Entamoeba* (132, 133). Both genera include benign and pathogenic species capable of causing debilitating disease in humans (132, 133). Subspecies genotyping connected outbreaks and differentiating levels in pathogenesis of keratitis (109). Touching on the first issue outlined by Maiden et al. 1998, typing schemes have been applied to *Acanthamoeba* species in order to track the spread of outbreaks by typing isolates and phylogenetically comparing them with previous outbreak associated isolates from Chicago (101, 109). Further to this idea, typing methods were applied to differentiate

between virulent strains of *E. histolytica* that cause disease and strains that do not originate from within the same geographic area (111). The study found that although *E. histolytica* individuals tended to display unique genotypes, some markers could determine the individual virulence (111). These type of studies indicate that molecular typing methods do work on amoebae for differentiating populations and virulence and that there is a strong link between the necessity to type species and the understanding of pathogenicity and disease within those genera (101, 109, 110, 128, 132, 134-136).

Protozoa in general, and amoeba in particular are proposed to contain some of the largest genomes which are presently known at between 200 and 600 times the size of the human genome (137). *N. pemaquidensis* has a genome of 43.7Mbp and the parasome ~ 9.5Mbp (66). Whole genome sequence information is currently lacking for *N. perurans* and related species. This can possibly be attributed to technological issues such as dealing with length heterogeneities, purity of DNA, differentiating between two genomes and the economic implications, as this technology is still expensive (66, 138). However, some partial sequence data from transcriptome analyses are available (84), and work into whole genome sequencing is currently being done on *N. pemaquidensis* (66). As this is not yet available, and in the absence of whole genome analysis, shorter read typing methods could provide valuable insight into the global epidemiology of this significant salmonid pathogen. For *N. perurans*, an ideal typing method would be sensitive enough to detect different population genotypes from an AGD outbreak, but diverse enough not to be compromised by differences between individuals within a population such as with the length heterogeneity in the ITS regions.

MLST analysis typically uses genes that code for proteins important for cellular function, also known as 'housekeeping genes' (101). Housekeeping genes, similar to the 18S rRNA gene, are robust against mutation which is important as mutations have the potential to interrupt the housekeeping genes' cellular functions which would cause negative effects on the cell (101, 102, 109, 139). Non protein coding genes are also used for MLST so long as they are similarly robust with a ratio of non-synonymous substitutions per non-synonymous site to synonymous substitution per synonymous site at less than one (102). MLST therefore is a good fit as it is sensitive enough to detect spontaneous changes within clonal cultures but also combines multiple genes, which circumvents unreadable sequencing in one gene (101, 103). Combining and comparing several conserved genes could potentially identify genotypes within a species, and inform how multiple genotypes with different traits, such as level of pathogenicity, could arise from a founding common ancestor (109). There are, however, difficulties with applying MLST to eukaryotes due to the diploid or polyploid nature of these organisms (140). For example, classical MLST software generally treat heterozygous or multi-state sites as ambiguous information and ignores them (140). For instance, in the cases of *Entamoeba* and *Acanthamoeba*, a classical MLST analysis was applied. In order to resolve the differences between samples, a sequence-by-sequence manual determination of ambiguous bases and sequence alignments required, since the software did not have algorithms to handle the discrepancies introduced by heterozygotes (109, 111). This need for manual sequence manipulation prior to software application is a limitation to this method that could lead to methodological differences, which may affect the between-lab reproducibility of the results.

MLSTest (MLST data analysis software) was developed in 2013 to solve these issues. It treats multi-state sites in two ways: 1) through average states which calculate the distance between multiple bases (i.e., Y) as the mean distance between all possible resolutions of the heterozygous base (i.e., C or T), or 2) through duplication of SNP and polymorphic sites while removing heterozygous states (140). MLSTest software has been applied to several pathogenic and parasitic eukaryotic species to study their epidemiology and population structure, including *Leishmania* spp. and *Trypanosoma cruzi,* but has yet to be tested on *Neoparamoeba/ Paramoeba* species (110, 111, 134).

MLST was applied to a variety of environmental and cultured (clonal and non-clonal) *N. perurans* samples from Canada, Ireland, Norway, Scotland, Tasmania, and USA. The study had two main objectives: 1) To apply a typing method to *N. perurans* samples to identify new genotypes, and 2) To examine if *N. perurans* samples form phylogenetic groupings based on their geographic origin.

2.2 MATERIALS AND METHODS

2.2.1 STRAINS AND DNA EXTRACTION

Three non-clonal samples of *N. perurans,* Tasmania 1, 2 and 3 were cultured from Atlantic salmon (*Salmo salar*) sampled during AGD outbreaks on various Atlantic salmon farms in Tasmania or reisolated from experimental challenges of Atlantic salmon. Specifically, Tasmania 2 was collected from sacrificed high parasite load, pre-bath Atlantic

Salmon from the Huon Aquaculture Company in 2015. Tasmania 1 and 3 were taken from the maintained infection tanks at the University of Tasmania across 2014/2015. Tasmanian clonal cultures were first isolated in 2010 and separated into clonal lines C4 and C8, clones C8a and C4a-d are subcultures from those original clonal lines. Clonal samples were isolated from cultures on 35 ppt seawater malt yeast agar plates incubated at 18°C with a marine bacterial overlay. All samples were stored at -20°C in RNA preservation solution (4 M ammonium sulphate, 25 mM sodium citrate, 10 mM EDTA; pH 5.2). In addition to samples from Tasmania, samples of *N. perurans* were received from Ireland, Scotland, Norway, USA and Canada in a variety of preservation solutions (lysis buffer [4M urea, 1% SDS, 0.2M NaCl and 1mM sodium citrate], 96% ethanol and RNA preservation solution) and stored at -20°C (Table 2.1).

DNA was extracted following a modified protocol (141). Briefly, the samples stored in RNA preservation solution were centrifuged at 16,000xg for 10 min to pellet the cells. This step appeared to be essential in recovering DNA from samples stored in RNA preservation solution perhaps due to a change in cell density from the high salt content of the solution. The supernatant was removed, and the pellet was incubated with 500 µL lysis buffer (4M urea, 1% SDS, 0.2M NaCl and 1mM sodium citrate) for 10 min at 55°C with occasional vortexing. The tubes were then immediately placed on ice for 5 min before the addition of 250 µL ammonium acetate (7.5M). The tubes were vortexed for 20 s and then centrifuged at 14,000xg for 5 min at 18°C prior to precipitation. Once the supernatant was recovered in a new 1.7 mL tube, precipitation was achieved through the addition of one volume isopropanol (between 500µL and 750µL depending on volume of supernatant recovered) containing 20 µg mL^{-1} of co-precipitate, pink (Bioline) to allow for better visualization of the

pellet. The samples were inverted 40 times to ensure proper mixing and incubated for 1 h at room temperature (approximately 23°C). Incubation was followed by centrifugation at 16,000xg for 20 min. The resulting pellet was rinsed twice with 70% ethanol. The samples were centrifuged between washes and briefly after the final wash to ensure all the ethanol was removed. The pellet was resuspended in 50 µL elution buffer (10 mM Tris-Cl, pH 8.5). The samples stored in lysis buffer were processed following the same protocol except that the initial pelleting step was omitted. The vials were vortexed to homogenize the mixture, 500 µL transferred to a 1.7 mL tube, incubated at 55°C for the specified 10 min and the protocol followed as above.

The gill samples stored in ethanol were processed using a slightly modified protocol. The largest quantity of *N. perurans* was present in the ethanol used to store the gills rather than on the preserved gills. The DNA was then extracted from the ethanol-preserved amoebae following the same protocol used for the RNA preservation solution samples.

Duplicate samples were not included in the analysis. Duplicate samples were defined as samples that originated from the same isolation i.e. different sections of the same gill basket or subsamples of the same isolate or clone. The samples that were included in this analysis were the samples with the highest DNA yield and the least amount of sequencing inhibition.

Table 2.1 *Neoparamoeba perurans* samples listed by country of origin, samples name, year of isolation, the type of culture (clonal or mixed), the type of fixation used ('lab samples' indicate samples that were not preserved before DNA extraction), and the year that AGD outbreaks were first recorded for that country in literature.

Origin	Isolate	Year	Type	Fixation	Year of first recorded AGD outbreak (reference)
Tasmania, Australia	Tasmania C8a	In culture since 2010	Clonal	Lab Samples	1985 (41)
Tasmania, Australia	Tasmania C4a	In culture since 2010	Clonal	Lab Samples	
Tasmania, Australia	Tasmania C4b	In culture since 2010	Clonal	Lab Samples	
Tasmania, Australia	Tasmania C4c	In culture since 2010	Clonal	Lab Samples	

Tasmania, Australia	Tasmania C4d	In culture since 2010	Clonal	Lab Samples	
Tasmania, Australia	Tasmania 1	2014	Mixed Culture	RNA preservation solution	
Tasmania, Australia	Tasmania 2	2015	Mixed Culture	RNA preservation solution	
Tasmania, Australia	Tasmania 3	2015	Mixed Culture	RNA preservation solution	
Ireland	Ireland 1	2014	Mixed Culture	RNA preservation solution	1995 (47)
Ireland	Ireland 2	2015	Mixed Culture	RNA preservation solution	

Scotland	Scotland 1	2014	Mixed Culture	RNA preservation solution	2006 (142)
Scotland	Scotland 2	2014	Mixed Culture	RNA preservation solution	
Scotland	Scotland C	2014	Clonal	RNA preservation solution	
Washington, USA	USA	2015	Mixed Gill Isolation	Lysis Buffer	1985 (79)
British Columbia, Canada	Canada	2015	Mixed Gill Isolation	Lysis Buffer	2014 (30)
Norway	Norway	2014	Gill Arch	96% Ethanol	2006 (36)

2.2.2 SELECTION OF MLST LOCI

MLST literature (101, 109, 143) the initial selection of housekeeping genes was determined through Genbank searches. Eleven genes [adenosine kinase, alpha tubulin, beta tubulin, beta-actin, elongation factor 1, elongation factor 2, glyceraldehyde 3-phosphate dehydrogenase, glycogen phosphorylase, succinate dehydrogenase complex flavoprotein subunit A, Rab GTPase, and RNA polymerase 2 subunit] were chosen based on the number of available sequences from amoeba and other related organisms from which primers could be designed. Homology-based oligonucleotide primers were designed from available sequences in Genbank where possible or related organisms (*Neoparamoeba pemaquidensis, Neoparamoeba branchiphila, Naegleria* spp*., Acanthamoeba* spp*.* and *Entamoeba* spp*.*). Sequences were aligned using the Geneious version 8.1.6 software (144). Three primer pairs were generated for each gene using Geneious primer prediction and manually adjusted to cover polymorphic nucleotide sites with a bias towards *N. pemaquidensis* sequences. All primers were then tested with *N. perurans* genomic DNA and the top primer pair for each gene chosen based on length, GC content, coverage of polymorphic sites, and suitability to direct DNA sequencing.

From the initial list of eleven genes, five were discarded after initial tests due to lack of amplification of *N. perurans* DNA or for a lack of specificity. The top primer pairs of the remaining six genes [elongation factor 1 (ELF 1), elongation factor 2 (ELF 2), beta tubulin (β-tub), beta-actin (β-act), RNA polymerase large subunit 1(Rpb1), and succinate dehydrogenase complex flavoprotein subunit A (SDHA)] were used on all samples and used in sequencing. The final sense and antisense primers are shown in Table 2.2.

2.2.3 PCR PROTOCOLS

An initial PCR reaction was carried out on all samples confirming *N. perurans* as the sole isolated *Neoparamoeba spp.* using primers for *N. perurans* (44, 45), *N. pemaquidensis* (145) and *N. branchiphila* (59). The 10 µL PCR reaction consisted of 5 µL 2x MyTaqHS mix (Bioline, NSW, Australia), 500 nM of each primer, 2 µL water and 2 µL template, the amplification conditions: 3 min at 95°C, 35 cycles of 30 s at 95°C, 25s at 55°C and 10s at 72°C. The MLST PCR reactions were carried out in 20 µL reactions containing 10 µL 2x MyTaqHS mix (Bioline), 500 nM of each primer, 6 µL water and 2 µL genomic DNA template. Amplification conditions were as follows: an initial 3 min at 95°C, then 15 s at 95°C, 35 cycles of 30 s at [ELF2 – 64.5°C, ELF1, rpb1, β-tub, SDHA – 58.4°C and β-Act at 54.5°C], 15 s at 72°C, with a final extension of 1 min at 72°C. In some instances, one single band could not be resolved for some samples. In this case, bands of the targeted size were excised from the gel and

Table 2.2 MLST genes: elongation factor 1 (EF1), beta tubulin (β-tub), RNA polymerase large subunit 1 (Rpb1), beta actin (β-Act), elongation factor 2 (ELF2) and succinate dehydrogenase complex flavoprotein subunit A (SDHA) with the primer sequences and amplicon length of the fragments used in the analysis.

Gene	Primer Sequence 5'-3'	Amplicon Length (bp)
ELF1 Sense	AGAAGGAAGCCGCCGATATG	558
Antisense	GACAACCATACCGGGCTTCA	

ELF2 Sense	GAGGAGTACGCCCAAATCCC	480
Antisense	CCATAGATACCACCACGGGC	
Rpb1 Sense	GCTGAGGATCGACCCCAAAA	512
Antisense	CGCGACGTATCTCTGAAGCT	
β-Act Sense	CATCTATGAGGGTTATG	375
Antisense	GATGATCTTGATCTTCA	
β-Tub Sense	CTTTGTCCCTCCACCAGCTT	378
Antisense	CGCTGGACTTTTGTTGGAGC	
SDHA Sense	GGTGGTATTACTGGACGACAATCT	340
Antisense	GGCAGAGATTGGAAGGAA	

amplified by an additional PCR for 25 cycles. Amplified bands were purified using SureClean plus (Bioline) and directly sequenced in both directions (Macrogen, Seoul, Korea). Primers used in both sense and antisense sequencing were identical to primers used for amplification (Table 2.2).

2.2.4 DATA ANALYSIS

Using the Geneious alignment software, alignments were created for each individual gene fragment with the 16 selected samples. Heterozygotes were identified using a peak

similarity threshold of 90% to determine “real” heterozygotic and ambiguous sites. The forward and reverse sequences were then compared for each isolate to confirm the validity of the sequences.

The MLST data were analysed using the MLSTest software (Copyright © 2017 IPE) (140) with the objective of identifying geographic subtypes based on nucleotide diversity. Allelic profiles were created in which the MLSTest software calculated the Typing Efficiency (TE) for each allele. The TE is a representation of the number of identified genotypes within a gene divided by the number of polymorphic sites. The discriminatory power, or the probability that two strains can be differentiated based on that gene when pulled at random from a population, was also calculated using the MLSTest software (110).

Concatenated neighbour joining trees were created using both average states and SNP duplication to address heterozygous sites (140). Overall phylogenetic incongruences were addressed with the incongruence length difference test using the BIO-Neighbour joining method (BIONJ-ILD) with 1000 replications. This method is used to determine whether one or multiple fragments support a phylogeny that is not supported by the remaining fragments ($P<0.001$).

A basic BURST (Based upon related sequence types) analysis was run with the MLSTest program using a group definition of 6 shared alleles. The BURST and tree analysis are based on the concatenation of alignments (140).

2.3 RESULTS

2.3.1 PCR AMPLIFICATION AND SEQUENCING

The initial PCR reaction testing for the presence of *N. pemaquidensis* and *N. branchiphila* for all samples produced negative results for all DNA samples. PCR amplification of 6 gene fragments (elongation factor 1, elongation factor 2, RNA polymerase large subunit 1, beta actin, beta tubulin and succinate dehydrogenase complex flavoprotein subunit A) was applied to all 16 samples. The sequences are reported in GenBank as accession numbers (KX363875-KX363883). Reverse strand sequencing confirmed the sequences for all 16 samples across all 6 gene fragments with the exception of ELF1. The antisense strand could not be sequenced for the Ireland 1 and Norway samples due to insufficient material, so the sense strand sequences were used for the MLST analysis.

2.3.2 MLST ANALYSIS

The MLST analysis using concatenated neighbour joining trees gave a two-group pattern in the MLST graphical output, with the Tasmanian samples grouping separately from all other samples (Figure 2.1). Within the second group, Ireland and Scotland were grouped together as were Norway, Canada and USA. For the concatenated neighbour joining trees generated for all six loci the bootstrap values were slightly higher when using the 'single nucleotide polymorphism (SNP) duplication' method compared to the 'average states' method. When the 'average states' was applied, the Tasmanian samples group separately to all other geographic samples with a bootstrap of 62.8%. Ireland and Scotland were identified together within the second group (Scotland 1, Scotland 2, Ireland 2 and Scotland C) with 63.6% bootstrap support; Ireland 2 remained separate with 63.6% bootstrap

support. Norway, USA and Canada created a separate subgrouping with 62.8% bootstrap support (Figure 2.1). The same tree topology could be observed when concatenated neighbour joining trees were generated using SNP duplication instead of average states. Bootstrap support, however, changed with subgroup 1 (Ireland and Scotland) having 73.8% support, subgroup 2 (Norway, USA and Canada) having 73% bootstrap support (Figure 2.1. Values in blue). Though the two topologies were identical, the Incongruence Length Difference (ILD) was significant for the SNP duplication method (P = 0.0009) where it was not significant using the average states method (p=1). This factor combined with the fact that SNP duplication resolves differences by duplicating the bases and thus modifying the alignment, could have methodologically altered the bootstrap values. Therefore, the average states method was chosen as the most appropriate.

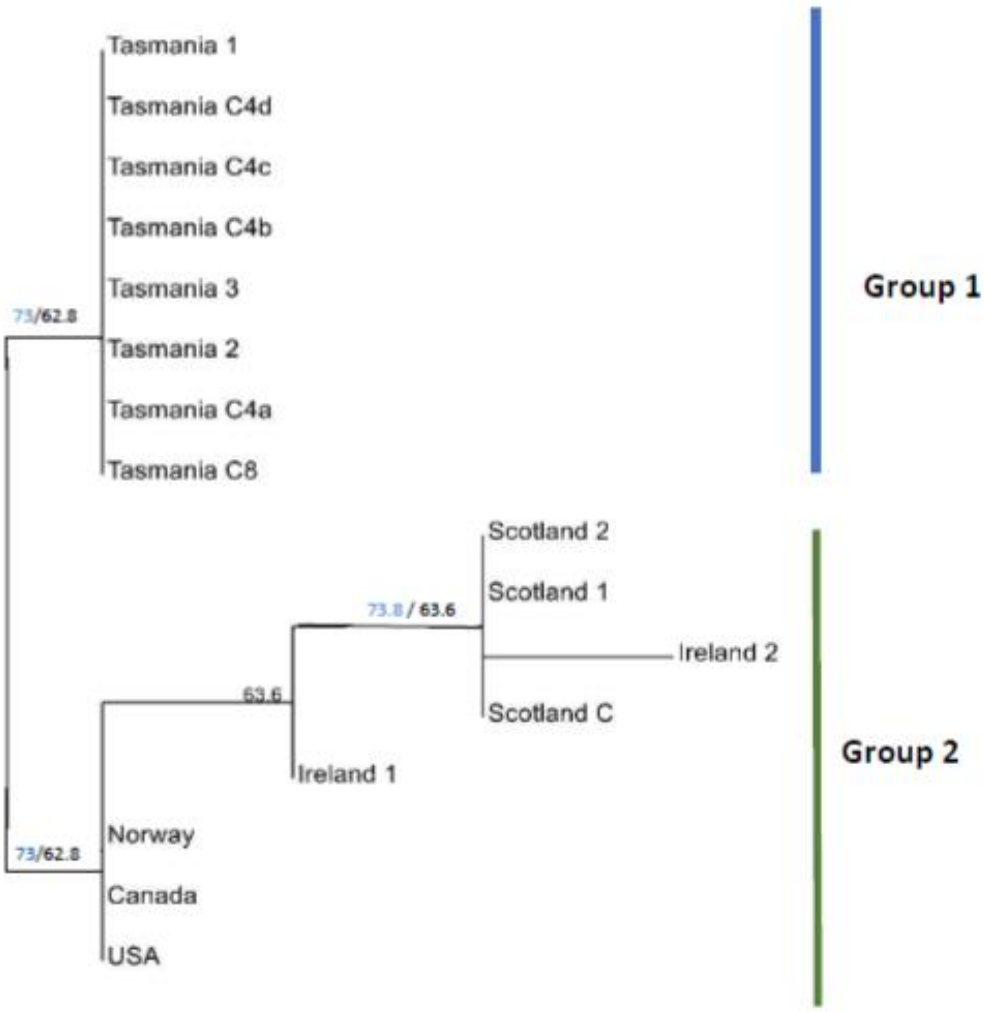

Figure 2.1 Concatenated Neighbour Joining Tree based on 6 MLST gene loci (elongation factor 1 (EF1), beta tubulin (β-tub), RNA polymerase large subunit 1 (Rpb1), beta actin (β-Act), elongation factor 2 (ELF2) and succinate dehydrogenase complex flavoprotein subunit A (SDHA)) using average states to resolve polymorphic sites. Different groupings are represented by vertical bars. Two distinct groups can be visualized; Group 1 (Tasmania) and Group 2 (Ireland, Scotland, USA, Canada and Norway). Branch support represent bootstrap values (1000 replications). Blue values represent the bootstrap support for the SNP duplication method (1000 replications).

Table 2.3 shows the allelic profile of the 6 loci analysed by the MLSTest software. There were very few polymorphic sites reported with the maximum for any given gene locus being 1. ELF1, RPB1 and SDHA showed the least discriminatory power (0.118) with one genotype and no polymorphic sites. Beta actin had the highest discriminatory power (0.588) though not the highest number of genotypes (ELF-2).

Table 2.3 *N. perurans* MLST targets showing the Typing Efficiency (TE) and Discriminatory Power (DP) for each gene loci [elongation factor 1 (EF1), beta tubulin (β-tub), RNA polymerase large subunit 1 (Rpb1), beta actin (β-Act), elongation factor 2 (ELF2) and succinate dehydrogenase complex flavoprotein subunit A (SDHA)] along with the relative variability of each gene.

Gene loci	No. of Genotypes	No. of Polymorphic sites	Typing Efficiency	Discriminatory Power
B-Act	2	1	2	0.588
B-Tub	2	1	2	0.228
ELF1	1	0	Infinite	0.118
ELF2	3	1	3	0.551
Rpb1	1	0	Infinite	0.118
SDHA	1	0	Infinite	0.118

2.3.3 BURST ANALYSIS

The burst analysis resolved 5 sequence types (ST): 1(all samples from Tasmania, including clones), 2 (Ireland 1), 3 (all samples from Scotland), 4 (Ireland 2) and 5 (USA, Canada and Norway). All the sequence types were related to each other and there was no predicted founder (Figure 2.2).

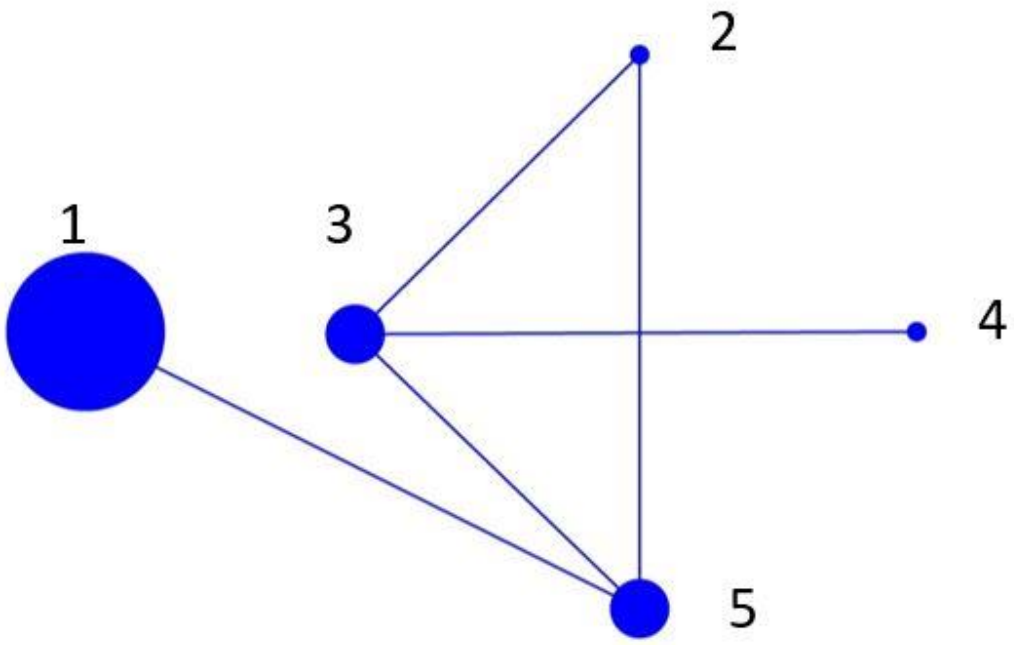

Figure 2.2 BURST graph of all sequences used in the MLST analysis. There were 5 sequence types defined: 1 (Tasmanian samples), 2 (Ireland 1), 3 (Scotland Samples), 4 (Ireland 2) and 5 (USA, Canada and Norway). The graph shows a connection between all samples typical of a clonal BURST configuration.

2.4 DISCUSSION

Based on MLST analysis, there were minor sequence dissimilarities between the geographically distinct *N. perurans* samples. When all 6 genes were combined, the 8 Tasmanian samples all grouped together separate from the rest of the geographically disparate samples. Moreover, all of the Tasmanian sequences were identical across all 6 genes, regardless of whether they were clonal or wild samples.

This grouping is not surprising, given that there is the greatest geographic distance separating Tasmania from the remaining locations (Ireland, Scotland, Norway, USA and Canada). However, the sequence uniformity could also indicate that there are potential geographic genotypes (146). Within the second 'non-Tasmanian' group (Scotland 1, 2 and clonal, Ireland 1 and 2, Norway, Canada and USA), there were few differences between samples. None-the-less, the MLSTest analysis resolved the Irish and Scottish samples grouping together, with sequence variation in the Irish samples. The isolate from Norway did not group with Ireland and Scotland but appeared to share a grouping with samples collected from the west coast of the USA and Canada.

The analysis showed few heterozygosities, and thus relatively low bootstrap support. Lower bootstrap support however, does not necessarily discredit the results as low bootstrap support is somewhat expected under the study conditions (147). Shorter sequence lengths are known to reduce bootstrap support, in a barcoding analysis in fungi reducing the sequence length caused the bootstrap support to fall below the significance cut-off while maintaining the same tree topology (148). The short sequences combined with the extremely low number of polymorphisms seen in this study may help explain why the

support is so low as few polymorphisms are also known to affect the bootstrap value (147, 148).

The small number of observed differences may be partly due to *N. perurans* being a marine microorganism. It has been postulated that the sheer number of individuals in any given microbial species are so large that dispersal would rarely be restricted by contrived geographical barriers (149). This postulation is amplified further when considering that *N. perurans* is a marine organism with few obstacles to geographic dispersal (15). For example, certain species of foraminifera (marine protozoa) have genetically identical samples collected from locations as disparate as the Arctic and Antarctic (149). Therefore, it is logical that we would find relatively low numbers of heterozygosities in any given *N. perurans* housekeeping gene.

Generally, MLST studies compare a high number of samples from one or two geographical locations (109), or from different hosts or locations within a host (111, 113, 134). Further, MLST studies on amoeba have focused on human parasitic genera, namely *Acanthamoeba* and *Entamoeba,* and as a result compare populations that are inherently more restricted from mixing with neighbouring populations (113). Nevertheless, the number of polymorphic sites we observed per gene per isolate differed from gene to gene following a similar trend to other MLST typing studies, but with considerably lower percentages (109, 111, 146). For instance, in a MLST study done on Acanthamoeba the number of variable sites for beta tubulin and elongation factor 1, were 23 and 4 respectively (determined from 40 samples of one known type)(109). The same genes produced 1 (beta tubulin) and 0 (elongation factor 1) variable sites determines from 2 samples from Ireland. This initial

analysis is promising. However, to understand whether these results are artefacts or indications that there are distinct genotypes associated with a specific geographical locality more samples will need to be examined. In particular additional clonal samples from the other geographic sites could be informative. Though environmental samples are ideal to get an accurate idea of the overall population, clonal samples help to resolve sequences where intragenomic variation occurs. There were a few instances of this occurring in the form of ambiguous base pairs (Appendix 3). It is envisaged that further work to incorporate more samples from existing AGD affected countries as well as from new and archival AGD outbreaks will further resolve potential genotypes and benefit the epidemiology of this important fish disease. This is discussed in more detail in chapter 5.

Unlike for the genera *Acanthamoeba* and *Entamoeba*, the MLST analysis showed little evidence of lineage development within *N. perurans* (109, 111). The BURST analysis further diminishes the evidence for highly genetically different geographic populations. The BURST graph showed connections between all sequences, and importantly, did not predict a primary founder. The primary founder denotes the genotype from which all subsequent genotype populations have descended (150). The lack of a primary founder is what would be expected within ubiquitous, asexually reproducing populations. On a global scale, there appears to be no “source” population to which subsequent specific geographic outbreak populations can be traced.

The genetic variation that we see is likely due to localized source populations. The development of aquaculture meant the introduction of year-round host populations creating conditions that are known to foster the development of new pathogens (151).

These local pathogen (i.e. *N. perurans*) populations exposed to very different conditions and selection pressures, would slowly differentiate despite the lack of physical barriers (152). Therefore, this study suggests that the increased disease outbreaks are not linked to the spread of specific virulent *N. perurans* strains from one initial outbreak site. The trend of expanding outbreaks is more likely to be correlated with changes in environmental conditions such as increasing global sea surface temperature (153), or increased farming pressure (27) providing favourable conditions for the development of pathogenicity on a regional scale.

Conclusion

This is the first study to use a typing method on geographic samples of *N. perurans* from gills of Atlantic salmon undergoing AGD infections. This study showed low levels of sequence heterogeneity using conserved gene sequences suggesting that these amoebae are closely related. The genetic variation that we see is likely due to localized source populations. The development of aquaculture meant the introduction of year-round host populations creating conditions that are known to foster the development of new pathogens. These local pathogen (i.e. *N. perurans*) populations exposed to very different conditions and selection pressures, would slowly differentiate despite the lack of physical barriers.

In addition, there was no predicted founder which indicates that there is no initial virulent population that is being spread, causing outbreaks. It is therefore proposed that the increasing number of AGD outbreaks worldwide is not due to transmission between locations but rather environmental factors. The trend of expanding outbreaks is more likely

to be correlated with changes in environmental conditions such as increasing global sea surface temperature, or increased farming pressure providing favourable conditions for the development of pathogenicity on a regional scale. It is likely therefore that there is little potential risk of transfer from farm site to farm site. Potential mitigation efforts therefore should be focused early detection based on known predictive conditions such as salinity and water temperature.

CHAPTER THREE: RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) ANALYSIS OF GEOGRAPHICAL SAMPLES OF *Neoparamoeba perurans*

3.1 INTRODUCTION

Only small sequence differences were found between the geographically diverse samples using MLST and a suite of six housekeeping genes (Chapter 2). There was not however, enough resolution to discern between samples and prescribe subtypes. It therefore would seem advantageous to select another typing method that would give a more complete understanding of the whole DNA genomic differences between samples.

The breadth of geographic outbreaks compared in this thesis is quite large (six countries) compared to similar MLST studies. Knowing intra-genomic micro heterogeneity occurs within *Neoparamoeba* (69, 80), characterization based on conserved sequences appeared ideal for initial analysis. The resulting MLST study showed few polymorphic sites across the genes. The outcome indicated that there may be the potential to geographically genotype the *N. perurans* strains however to better investigates this idea, a more polymorphic typing system that was not too labour intensive was desired. In contrast to the MLST which uses short segments of highly conserved or 'housekeeping' genes, RAPD takes into consideration the whole genome with the random binding of short repeat primers (101, 103, 116, 154).

RAPD has been successful in differentiating between both geographic samples and virulent subtypes in a variety of free living and opportunistic amoebae species, including *Naegleria, Entamoeba* and *Acanthamoeba* (128, 155-158). In a study comparing nineteen

geographically distinct *Acanthamoeba* isolates, the RAPD method successfully grouped the Brazilian isolates separate to the American reference strains (128). In a study done with *Naegleria*, RAPD was applied to isolates from New Zealand, Australia, Japan, France, Mexico and the United States. The survey was useful in separating isolates by their geographic origin but also showed diversity in isolates from the same countries (159).

The finer scale resolution of the RAPD method compared to MLST, given its use of broader genomic material, may provide further insight regarding the potential genotypes we observed using MLST analysis and elucidate potential differences that correlate with changes in virulence. Using more genomic DNA to further characterize *N. perurans* at the sub-species level could provide new information that may help us further understand major issues in AGD, particularly the increasing geographic distribution and frequency of outbreaks (80). This study re-examines the same sixteen samples used in the MLST study from Australia, Canada, Ireland, Norway, Scotland and the USA with a set of five RAPD primers. This allowed for the validation of RAPD as a method for characterizing the relationships between samples of *N. perurans,* and to investigate the potential of geographically linked genotypes.

3.2 MATERIALS AND METHODS

3.2.1 STRAINS AND DNA EXTRACTION

Samples were collected from six countries: Australia, USA, Canada, Ireland, Scotland and Norway (see Table 2.1, Chapter 2). The strains were isolated from Atlantic salmon sampled during AGD outbreaks on various salmon farms. The Tasmanian clonal samples were isolated on 35ppt seawater malt yeast agar plates that had been incubated at 18°C with a marine bacterial overlay and subcultured since 2010. The Australian samples were stored in RNA preservation solution (4 M ammonium sulphate, 25 mM sodium citrate, 10 mM EDTA; pH 5.2). In addition to samples from Australia, *N. perurans* samples were received from Ireland, Scotland, Norway, USA and Canada in various forms of fixation (Chapter 2). DNA was extracted as outlined in chapter 2 following a modified protocol from Bridle et al. 2015 (141). After the MLST analysis and prior to the RAPD analysis, the extracted DNA was stored at -20°C.

3.2.2 PCR AMPLIFICATION

Five 10-nucleotide primers were chosen: A1 (5'CAGGCCCTC3'), A15 (5'TTCCGAACCC3'), B10 (5'CTGCTGGGAC3'), B12 (5'CCTTGACGCA3') and B18 (5'CCACAGCAGT3'). These primers were previously reported in a study on the free-living amoeba *Naegleria fowleri* (115). Each PCR reaction mixture was made using MyTaq (Bioline) which contains a buffer, Magnesium chloride ($MgCl_2$) and dNTPs. Previous studies have indicated that changes in the concentrations of PCR buffer, $MgCl_2$, primer and DNA template concentrations can introduce variation into RAPD analysis. MyTaq was used to ensure consistent concentrations of the above. In addition, primer concentrations remained constant and replicates of the same DNA template was used for each PCR analysis.

For each reaction, the final volume was 20 µL and consisted of 10 µL My Taq HS (10 µM), 1 µL of primer (10 µM), 7 µL distilled water and 2 µL DNA template. For each primer two PCRs were run for maximum band discrimination. The initial PCR was run using the following protocol: an initial step of 2 min at 95°C to initialize the hot start, followed by a denaturation step of 15 s at 95°C, then 35 cycles of 30 s at 40°C, 15 s at 72°C, with a final extension of 1 min at 72°C.

Following the initial PCR reaction, a 1 in 50 dilution was made from the PCR products using distilled nuclease free water. The PCR product dilutions for each sample were then run a second time using the following PCR protocol: an initial 2 min at 95°C, followed by 15 s at 95°C, then a reduced number of cycles using 30 instead of 35 each 30 s at 40°C and 15 s at 72°C, with a final extension of 1 min at 72°C. Each primer and sixteen samples were run in triplicate following the above protocols.

3.2.3 GEL ELECTROPHORESIS

The products for each primer from all PCR runs were visualized through gel electrophoresis to gauge the reproducibility of the analysis. Each gel was made manually using 210 mL of 1x TBE buffer, 3.15 grams of molecular grade agarose for a 1.5% gel and stained with 10 µL RedSafe (iNtRON Biotechnology Inc., South Korea). The gels were run using the sub-cell® GT Cell system (Bio-Rad) at 150 volts for 100 min. The gels were then visualized using a UVP GelDoc-It Imager.

3.2.4 POTENTIAL CONTAMINATION MITIGATION

Due to the nature of the received samples, it was not possible to ensure that no bacterial contamination was present. In previous work with RAPD, it was demonstrated that for contaminating DNA to have an effect on the RAPD analysis it would have to be in large proportions compared to the target organism DNA within the sample (160, 161). To investigate if bacterial contamination might have an effect on the RAPD analysis, two approaches were taken. Firstly, as a majority of the samples received were preserved and thus bacteria could not be cultured, samples were qPCR tested using universal bacterial 16S rRNA, 27F and 518R primers. Second, the selected RAPD primers were applied to the bacterial supernatant of two laboratory *N. perurans* cultures from Tasmania that were used in the MLST and RAPD analysis - C4a and C8a. The protocol was as follows.

Bacterial culture overlay was collected from the surface of two agar *N. perurans* cultures and transferred into a 15 mL tube. The tube was vortexed to break up bacterial rafts that may have trapped amoebae cells and then centrifuged at 600xg for 10 min to pellet amoeba cell. The top 2 mL of liquid was removed to a sterile petri dish and observed under light microscopy at 10x and 40x magnification for the presence of amoeba and bacteria. The 2 mL was then transferred to a new 15 mL tube and again vortexed and centrifuged at 600xg for an additional 10 min. The top 1.5 mL was pippeted to a sterile petri dish and checked again to ensure that only bacterial cells were present. Once checked the sample was transferred to a sterile 1.7 mL tube and centrifuged at 16,000xg for 10 min to pellet the bacterial cells. The supernatant was removed, and an isopropanol DNA extraction

was performed on the pellet following the procedure outline in chapter 2 with the addition of 5 µL proteinase K during the 10 min incubation in 500 µL lysis buffer.

A PCR reaction was run to ensure the absence of amoebae cells using *N. perurans* specific primers NYF (5' ATCTTGACYGGTTCTTTCGRGA 3') and NYR (5'ATAGGTCTGCTTATCACTYATTCT 3') (15, 24). The following protocol was used: 95°C for 2 min, followed by 35 cycles of 95°C for 15 s, 55°C for 30 s, 72°C for 15 s before a final step of 72°C for 60 s. The extracted bacterial DNA was then run using the RAPD primers in triplicate following the same protocol reported above (Section 4.4.2).

The results of both PCR were visualized using 1.5 % agarose gel (45 mg molecular grade agarose and 30 mL 1xTBE buffer) stained with 3 µL RedSafe (iNtRON Biotechnology Inc., South Korea). The gels were run using the sub-cell® GT Cell system (Bio-Rad) at 100 volts for 1 hour. The gels were then visualized using a UVP GelDoc-It Imager.

3.2.5 PHYELPH ANALYSIS

Dendrograms depicting the relatedness of the samples were created using the PyElph program (162). The gels from all three replicates for each primer were visually compared for consistency in banding patterns for each isolate across all replicates. The images were compared side by side for discrepancies such as missing bands, additional bands or changes in position or pattern due to migration. Once this was completed, each gel was also compared using the PyElph program to ensure that nothing was missed using the previous visual comparison. The PyElph program detected the lanes, migration bands and molecular weights; manual corrections were made where necessary as with unavoidable gel

defects such as smiling. As with the visual comparison, the PyElph analyses for each replicate were compared to ensure that the banding patterns were nearly identical. A representative gel image for each primer was then uploaded into the program to create the matrix. Bands that appeared in only one of the three replicates were discarded and not used in either the individual or combined analysis. A similarity matrix was created through the program using the dice coefficient (see Appendix 2, 162). The dice coefficient was applied to presence/ absence data using the formula below. Where |X| and |Y| are the numbers of band in each sample (163).

$$s = \frac{2|X \cap Y|}{|X| + |Y|}$$

The values are then used by the PyElph program to construct a neighbour joining dendrogram for each primer (Figure 3.1).

3.2.6 STATISTICAL ANALYSIS

A Jaccard's coefficient distance matrix was constructed for the combined primer profiles by marking the presence or absence of a band as 1/0. The replicates were compared and bands that appeared in only one of the three replicates were discarded and not used in the combined analysis. The Jaccard coefficient is similar to the dice coefficient and measure, the similarity or dissimilarity between a large number of samples. The equation is below. In this case the number of bands in each sample are denoted A and B.

$$J(A,B) = \frac{|A \cap B|}{|A \cup B|} = \frac{|A \cap B|}{|A| + |B| - |A \cap B|}$$

Similarity and the inverse dissimilarity for each isolate was computed using the XLSTAT add on for Microsoft Excel (164). The dissimilarity matrix was then inputted into the Trex - online web program under the neighbour joining option taken from Saitou and Nei (165) and a dendrogram of the data was produced (166).

3.3 RESULTS

The RAPD profiles across all five primers were highly polymorphic with consistent and reproducible banding patterns over all replicates (Figures 3.8- 3.12). A total of 81 scorable bands were generated over all 5 primers.

Results from the RAPD analyses with all five primers were combined and Jaccard's coefficient was used to calculate a dissimilarity distance matrix (Table 3.1). The matrix was then used to construct a neighbour joining dendrogram with 1000 bootstrap replicates. There were several distinct groupings visualized with all Australian wild samples grouped together and all Australian clonal isolates grouping together albeit separate to each other. The Scottish non-clonal samples were also seen grouped together without the clonal isolate (Figure 3.1).

The dendrograms created for each for each primer showed variation across samples and primers with a few geographical consistencies (Figures 3.2-3.6). Primer B10 showed the highest level of geographic grouping with all Australian samples as well as all Scottish Samples grouping together (Figure 3.4). When all primer dendrograms are compared, there are a few consistent groupings. Norway and Canada grouped together when primers A15, B10 and B18 were used and the Scottish and Irish samples grouped together with some

isolate variation in every primer dendrogram.

The results from the bacterial contamination checks showed very low levels using the 16S rRNA, 27F and 518R primers and the RAPD run showed very few bands overall (Figure 3.7). The bands that were present in all bacterial RAPD replications were excluded from both the PhyELPH and Jaccard's analyses.

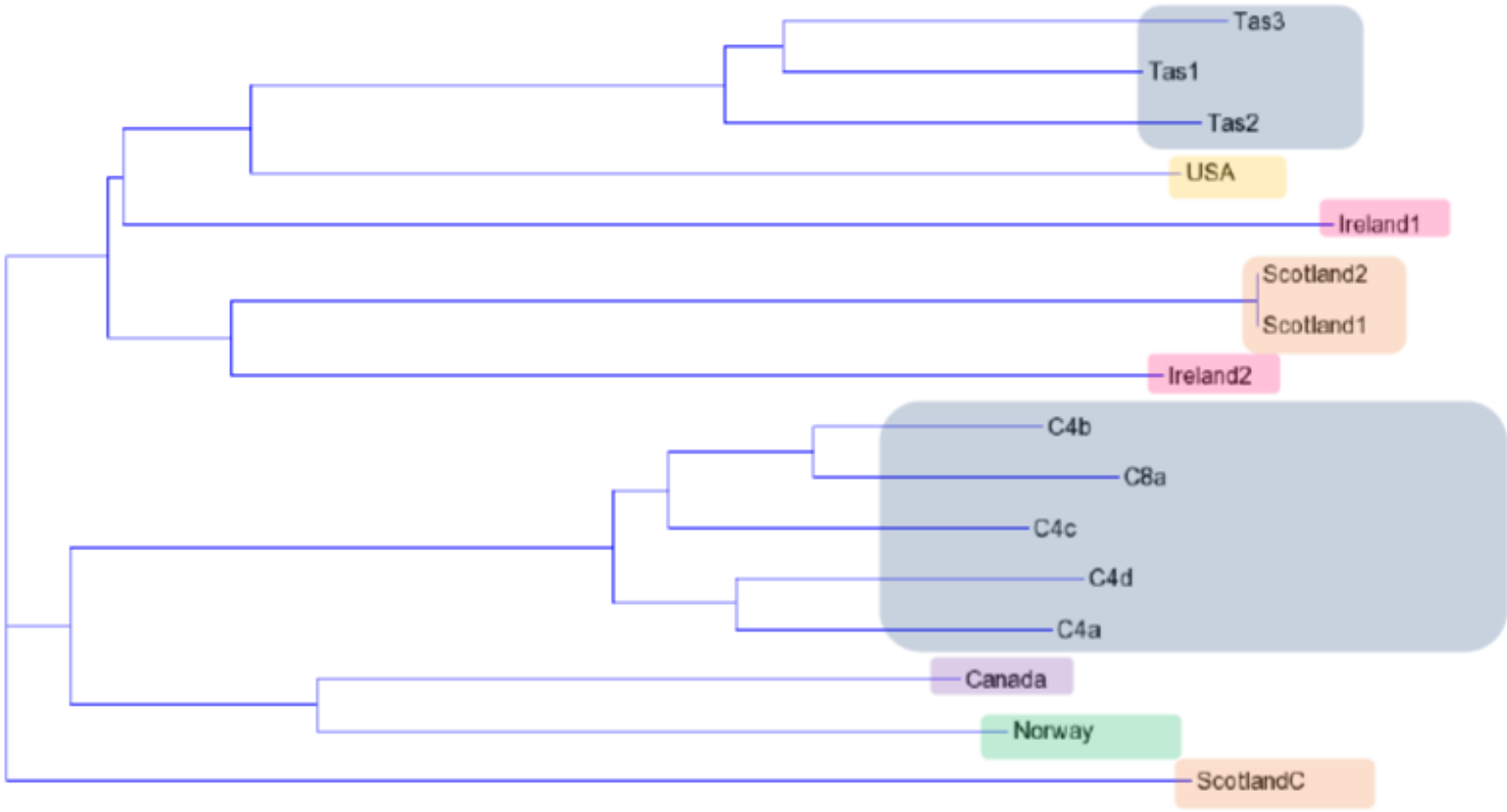

Figure 3.1 Dendrogram created from the Jaccard's coefficient distance matrix for the combined RAPD primer profiles (A1, A15, B10, B12 and B18). Colours represent geographic locations (Australia - blue, Scotland - red, Ireland - pink, USA - orange, Canada - purple and Norway - green).

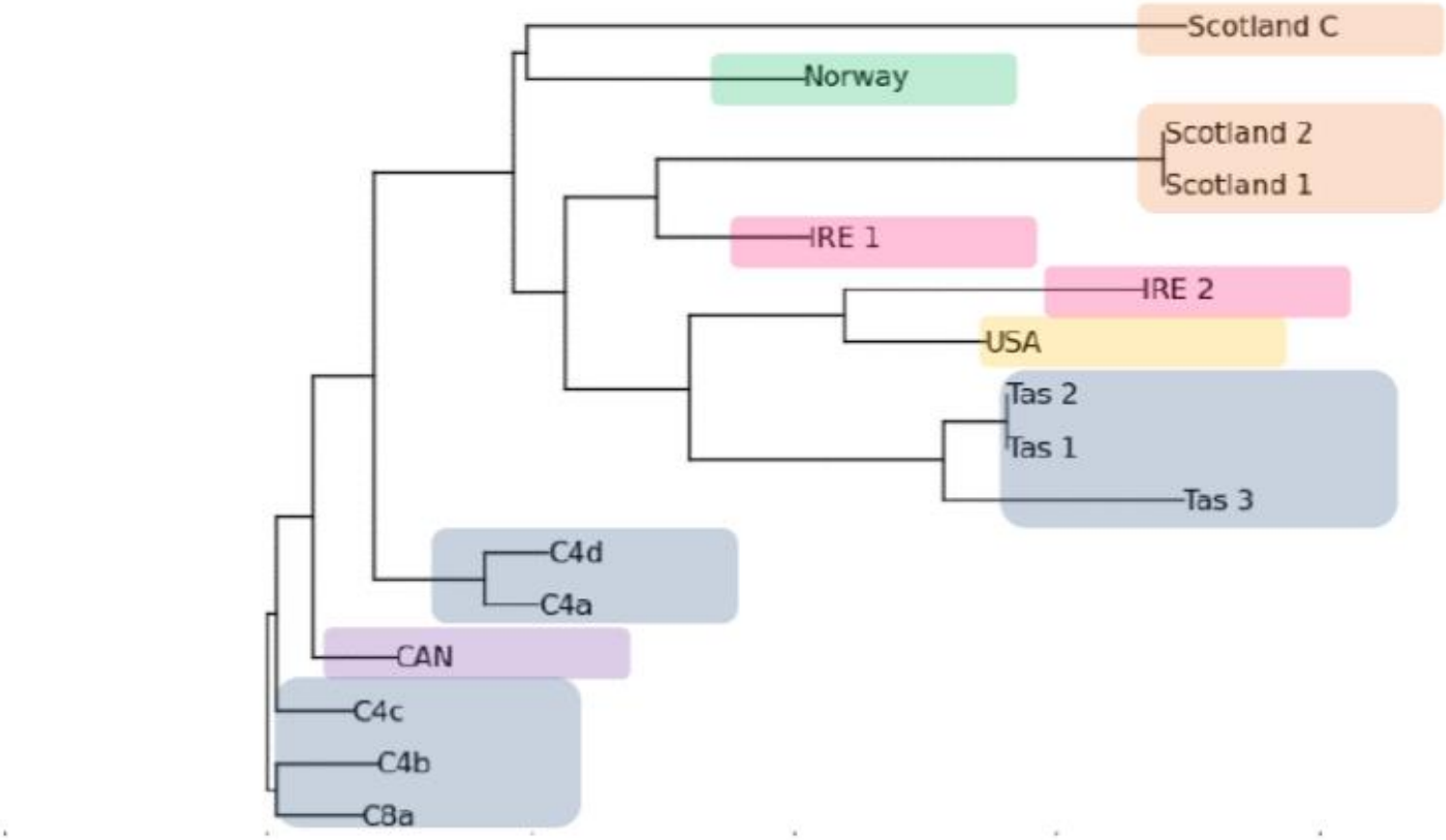

Figure 3.2 PyElph neighbour joining dendrogram of *N. perurans* samples for primer A1. Colours represent geographic locations; blue, Australia; red, Scotland; pink, Ireland; orange, USA; purple, Canada; green, Norway.

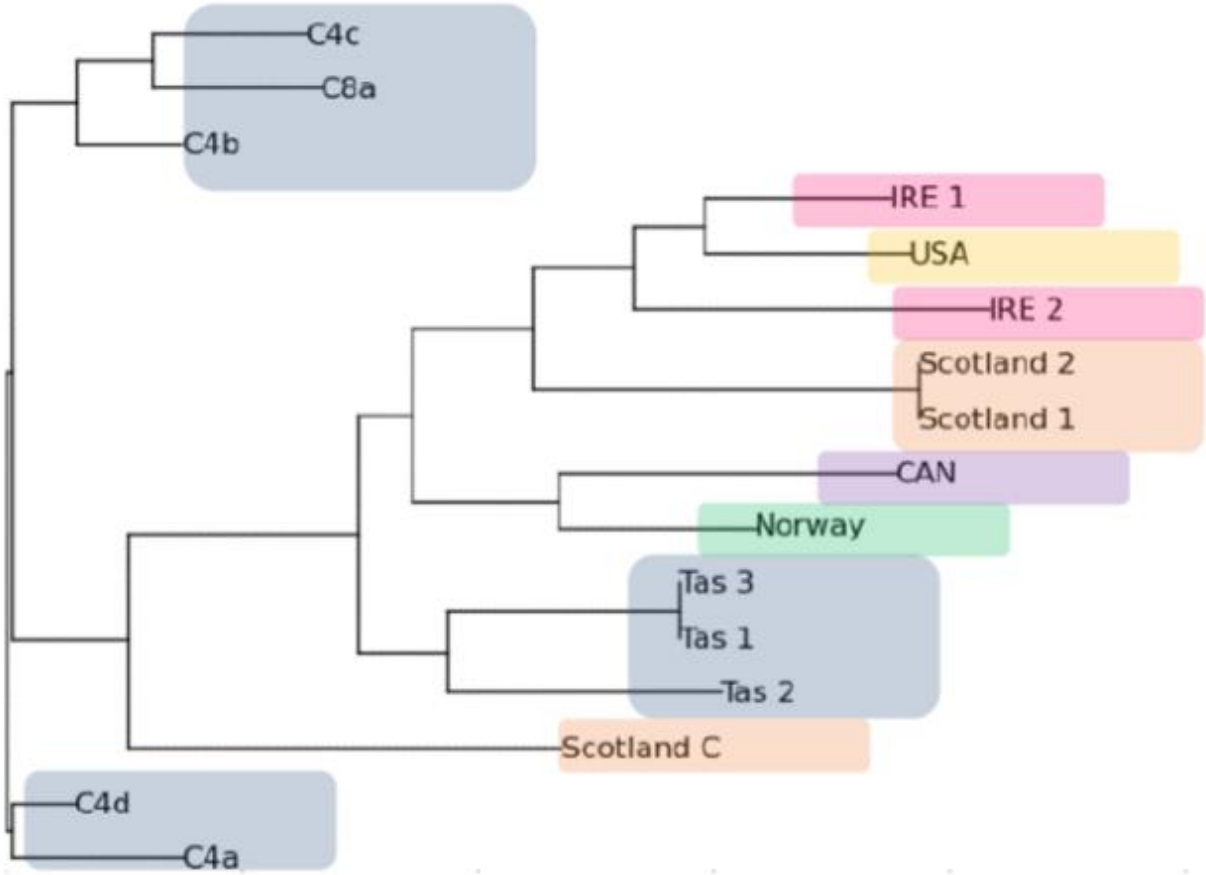

Figure 3.3 PyElph neighbour joining dendrogram of *N. perurans* samples for primer A15. Colours represent geographic locations; blue, Australia; red, Scotland; pink, Ireland; orange, USA; purple, Canada; green, Norway.

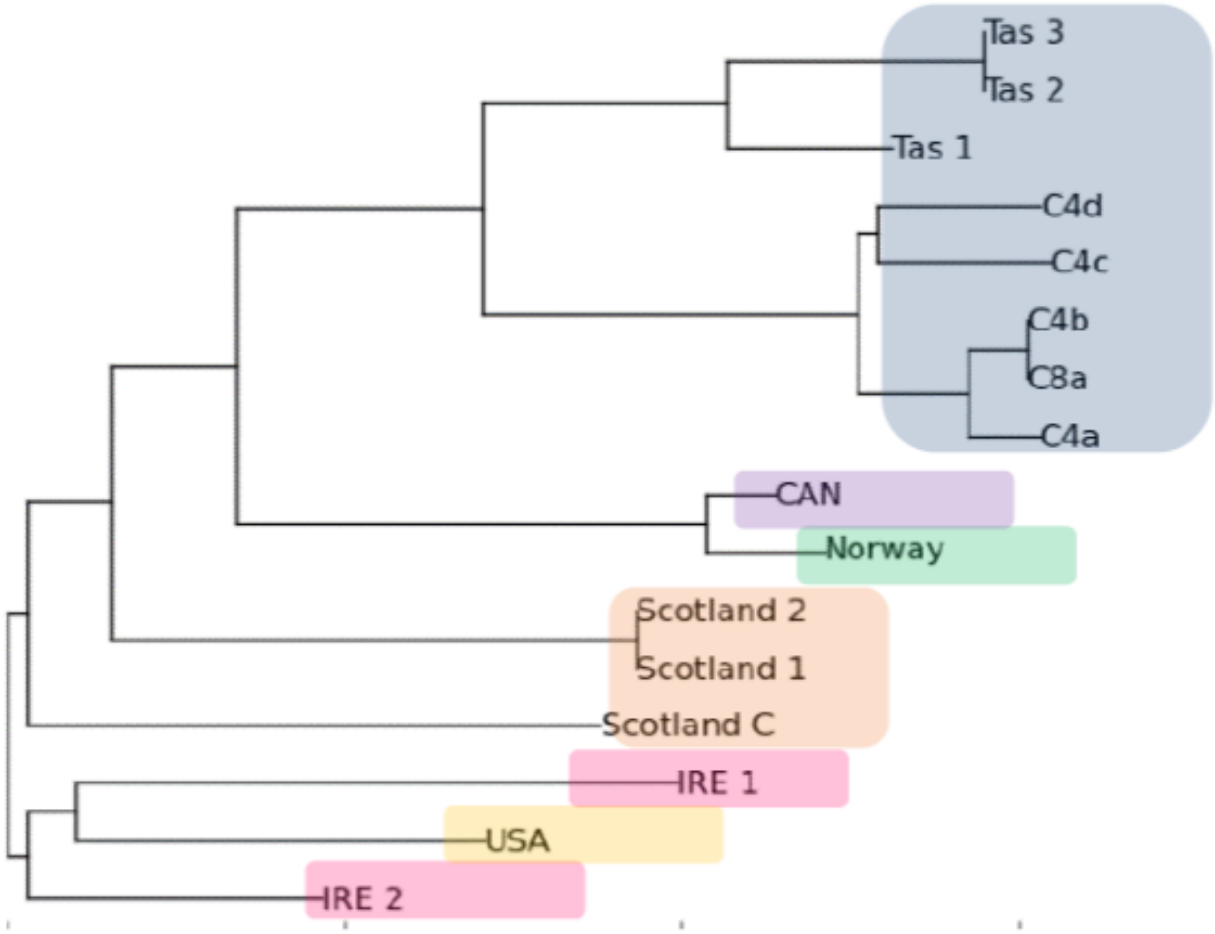

Figure 3.4 PyElph neighbour joining dendrogram of *N. perurans* samples for primer B10. Colours represent geographic locations; blue, Australia; red, Scotland; pink, Ireland; orange, USA; purple, Canada; green, Norway.

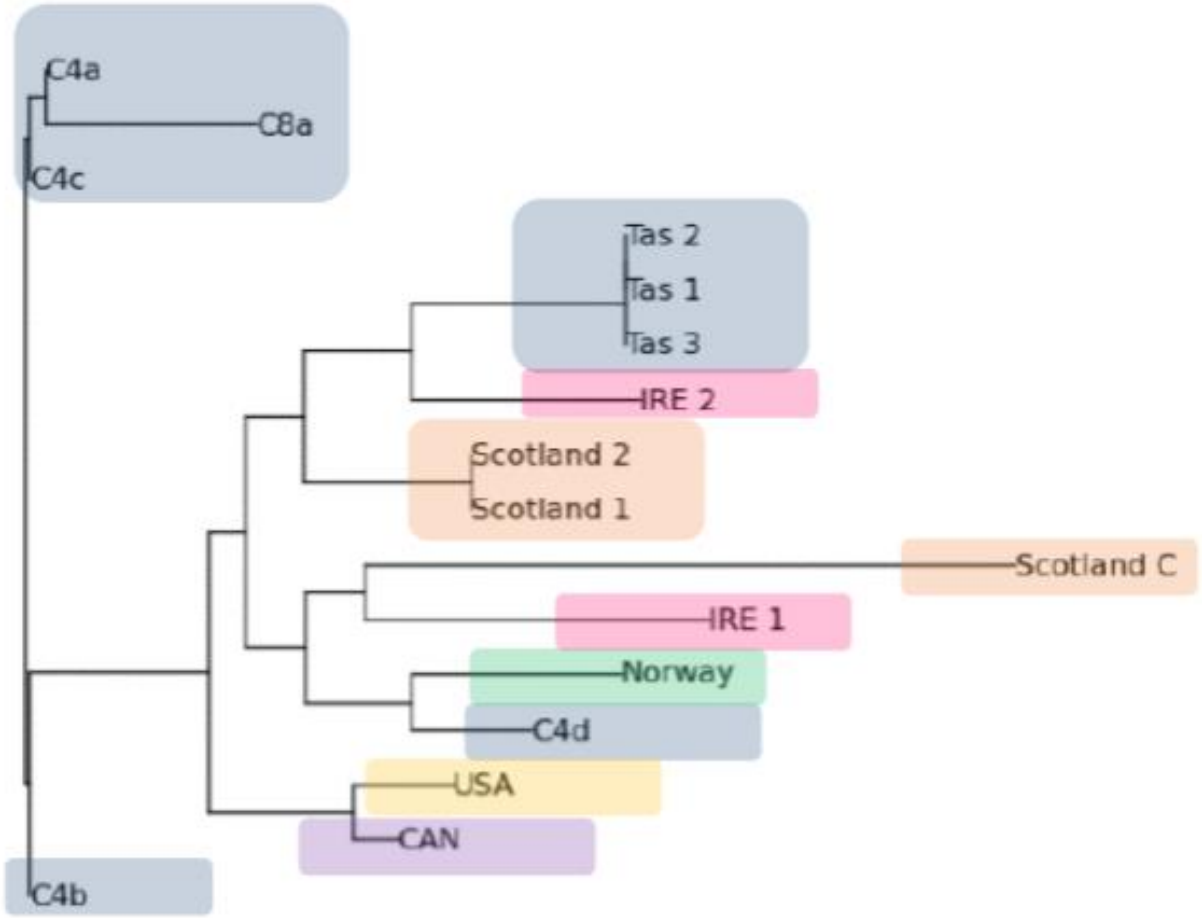

Figure 3.5 PyElph neighbour joining dendrogram of *N. perurans* samples for primer B12. Colours represent geographic locations; blue, Australia; red, Scotland; pink, Ireland; orange, USA; purple, Canada; green, Norway.

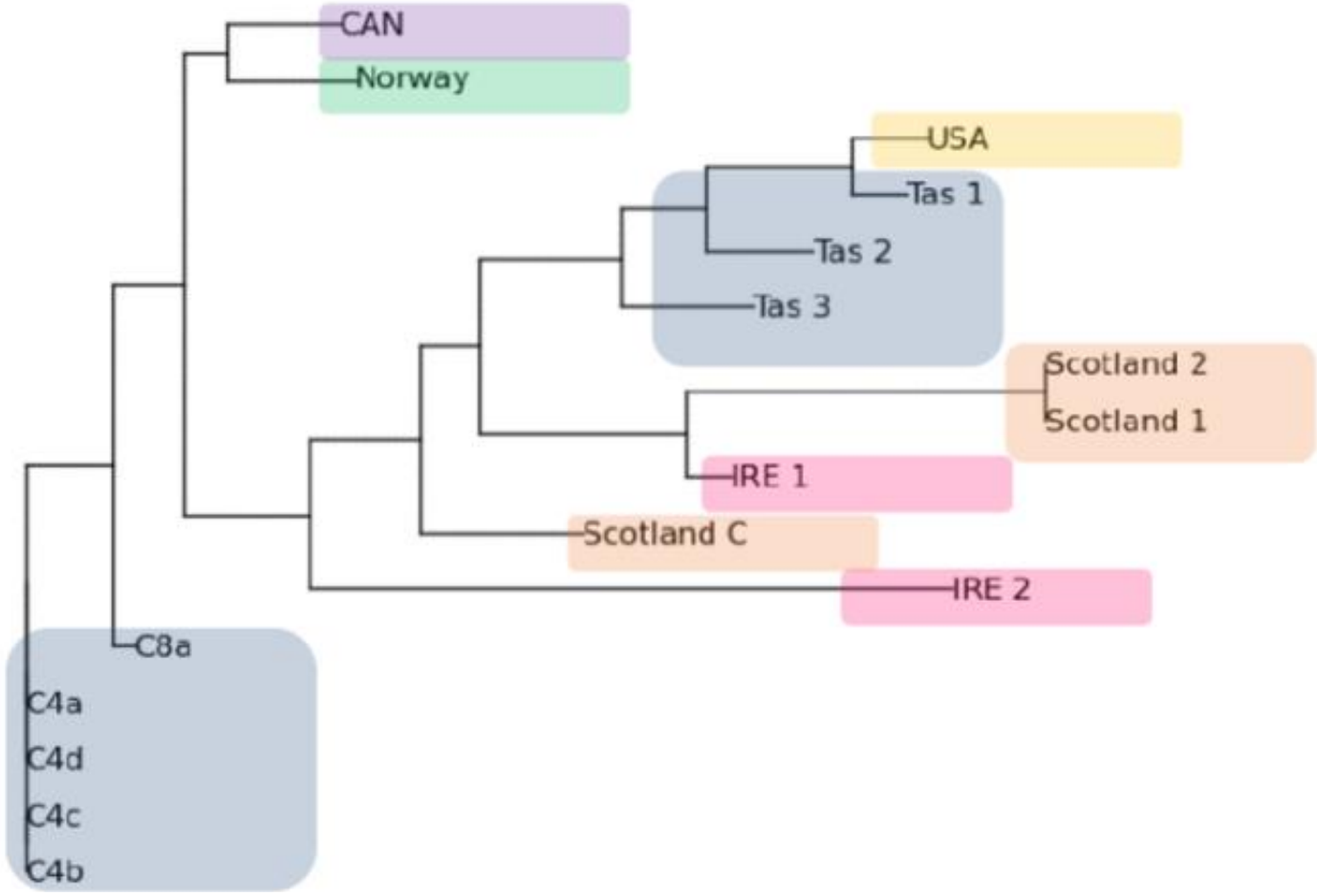

Figure 3.6 PyElph neighbour joining dendrogram of *N. perurans* samples for primer B18. Colours represent geographic locations; blue, Australia; red, Scotland; pink, Ireland; orange, USA; purple, Canada; green, Norway.

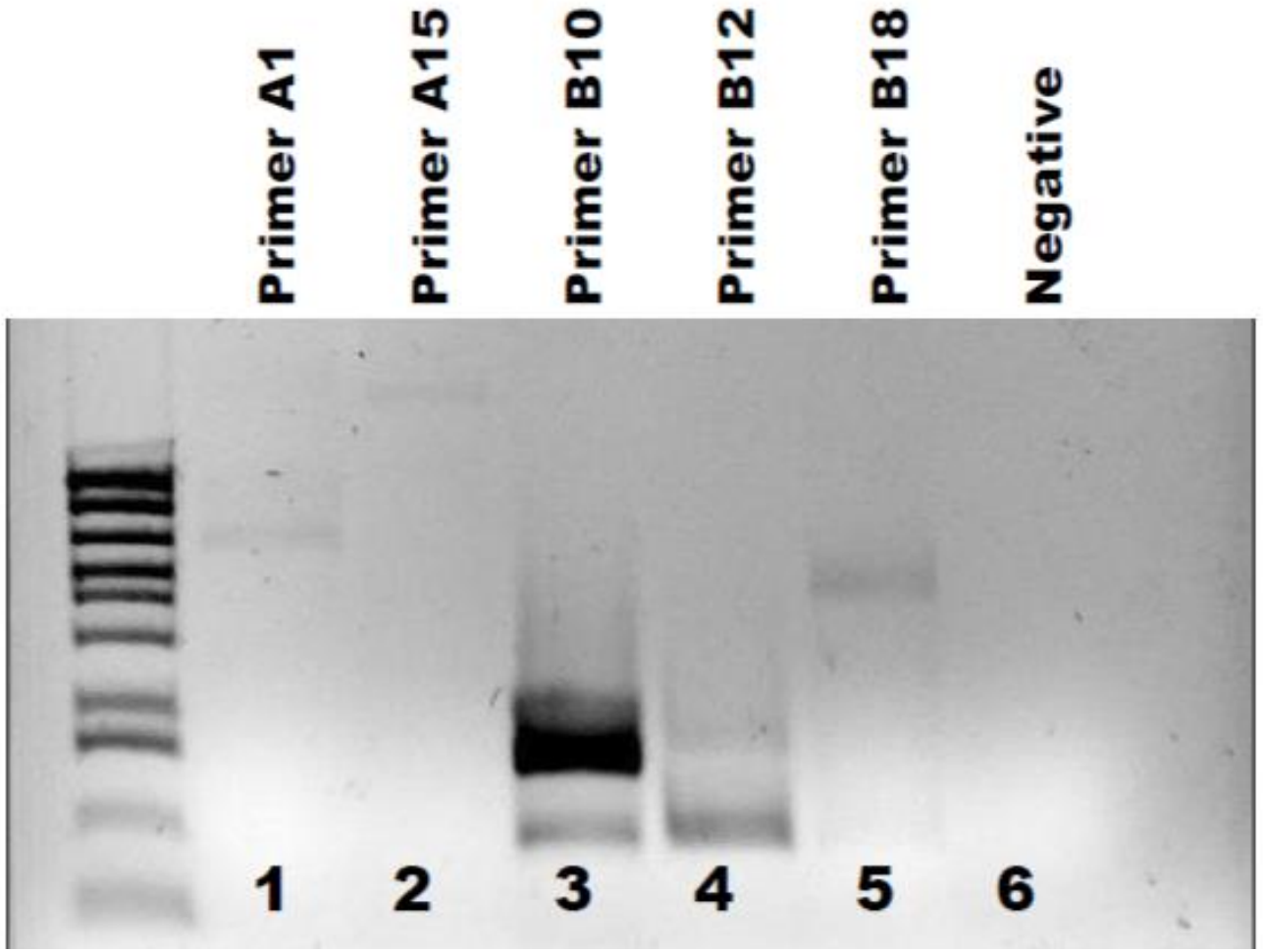

Figure 3.7 Agarose gel of amplified bacterial DNA from *N. perurans* culture C4a using RAPD primers: 1, A1; 2, A15; 3, B10; 4, B12; 5, B18; 6, Negative control.

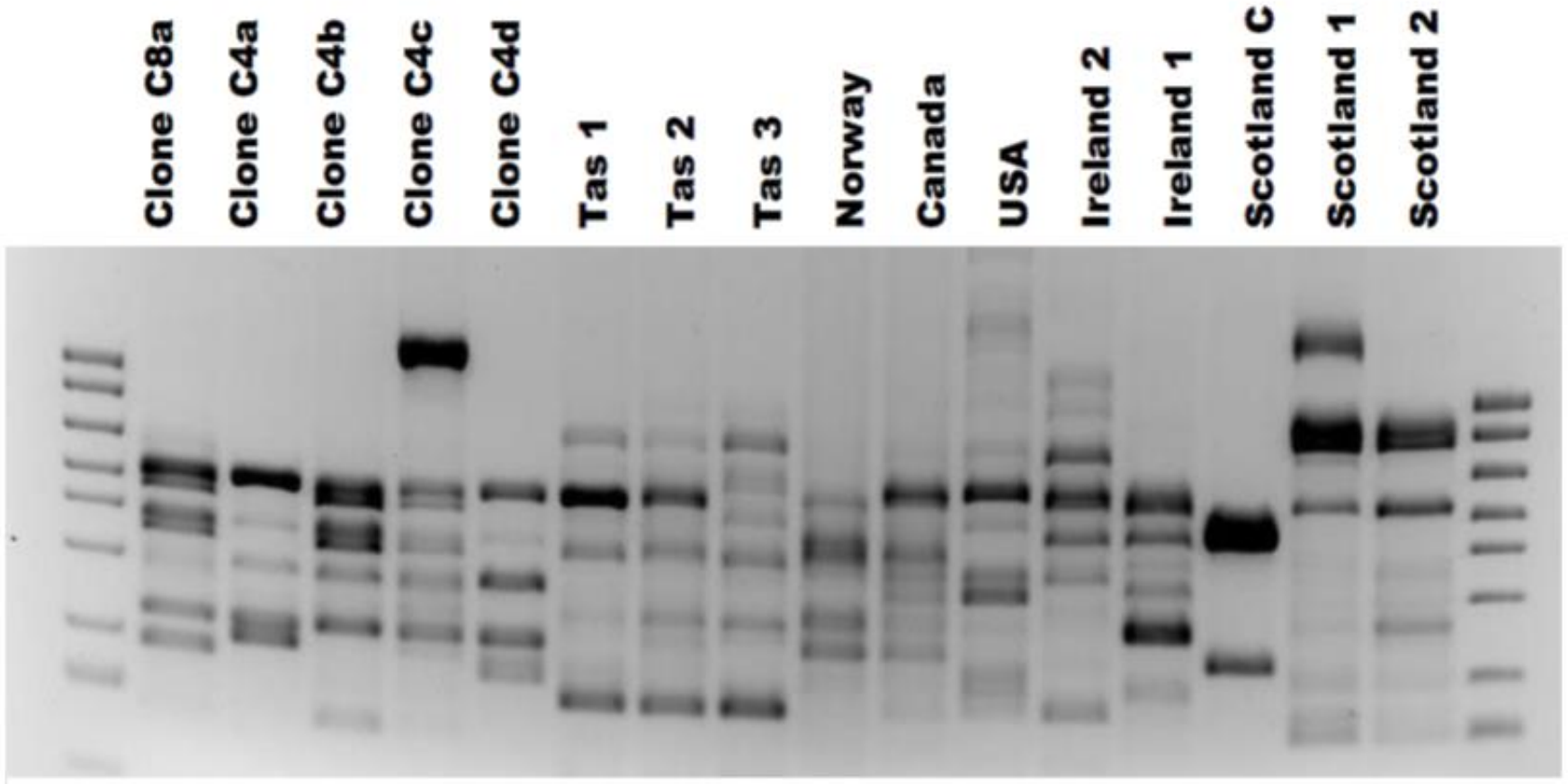

Figure 3.8 Agarose gel of amplified RAPD products for primers A1. 1, Clone C8a; 2, Clone C4a; 3, Clone C4b; 4, Clone C4c; 5, Clone C4d; 6, Tas 1; 7, Tas 2; 8, Tas 3; 9, Norway; 10, Canada; 11, USA; 12, Ireland2; 13, Ireland1; 14, Scotland clone; 15, Scotland 1; 16, Scotland 2.

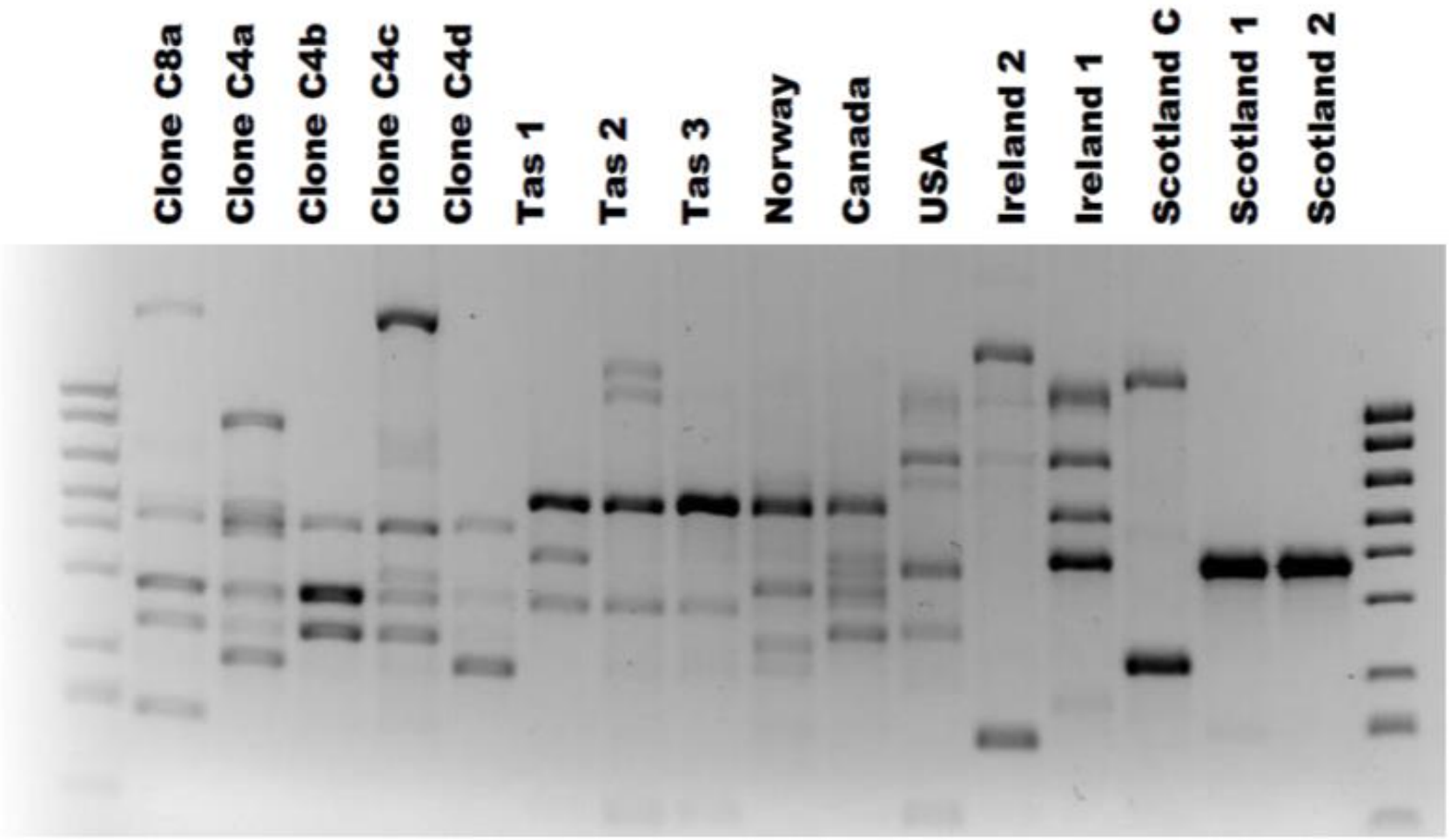

Figure 3.9 Agarose gel of amplified RAPD products for primers A15. 1, Clone C8a; 2, Clone C4a; 3, Clone C4b; 4, Clone C4c; 5, Clone C4d; 6, Tas 1; 7, Tas 2; 8, Tas 3; 9, Norway; 10, Canada; 11, USA; 12, Ireland2; 13, Ireland1; 14, Scotland clone; 15, Scotland 1; 16, Scotland 2.

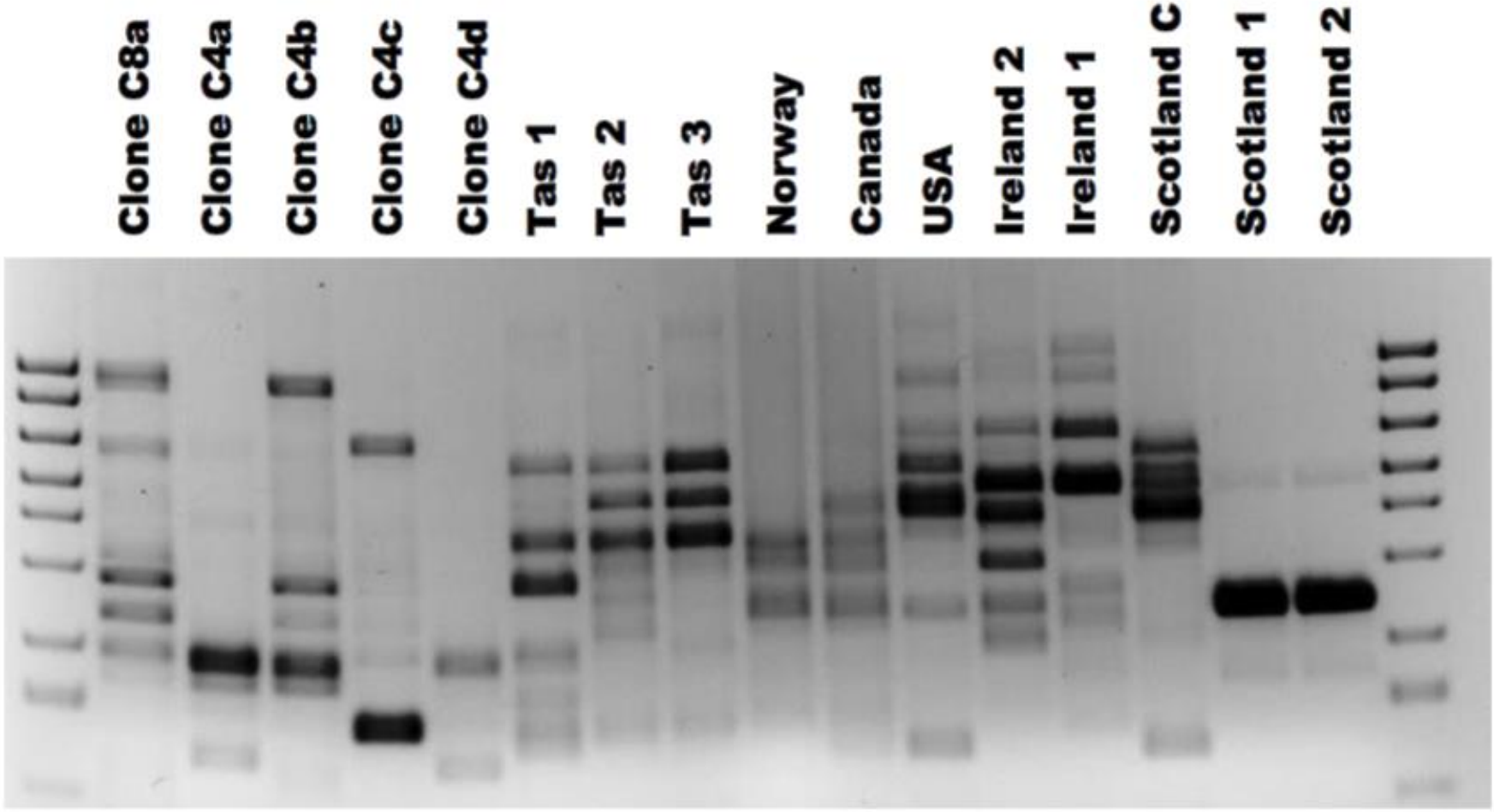

Figure 3.10 Agarose gel of amplified RAPD products for primers B10. 1, Clone C8a; 2, Clone C4a; 3, Clone C4b; 4, Clone C4c; 5, Clone C4d; 6, Tas 1; 7, Tas 2; 8, Tas 3; 9, Norway; 10, Canada; 11, USA; 12, Ireland2; 13, Ireland1; 14, Scotland clone; 15, Scotland 1; 16, Scotland 2.

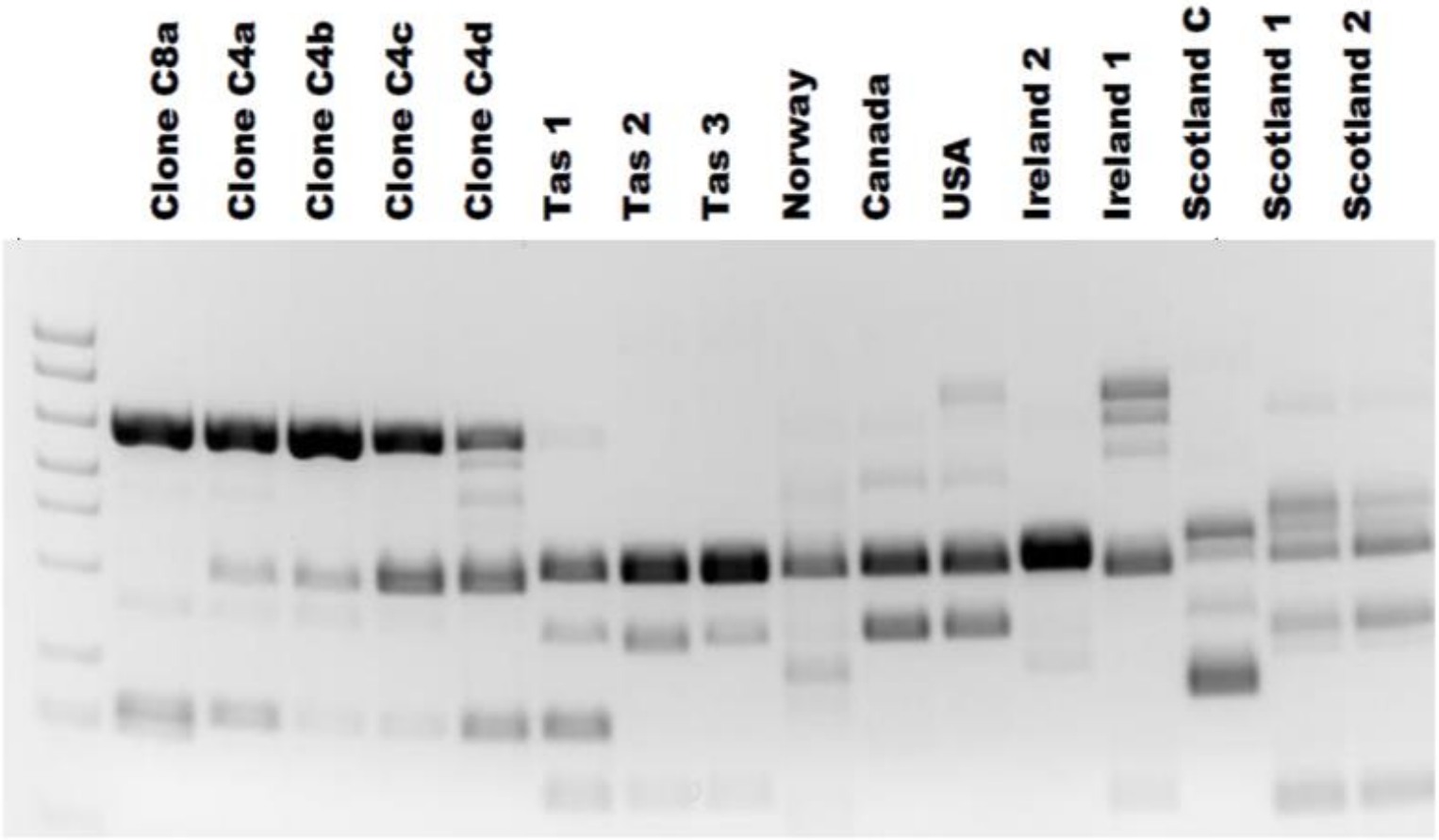

Figure 3.11 Agarose gel of amplified RAPD products for primers B12. 1, Clone C8a; 2, Clone C4a; 3, Clone C4b; 4, Clone C4c; 5, Clone C4d; 6, Tas 1; 7, Tas 2; 8, Tas 3; 9, Norway; 10, Canada; 11, USA; 12, Ireland2; 13, Ireland1; 14, Scotland clone; 15, Scotland 1; 16, Scotland 2.

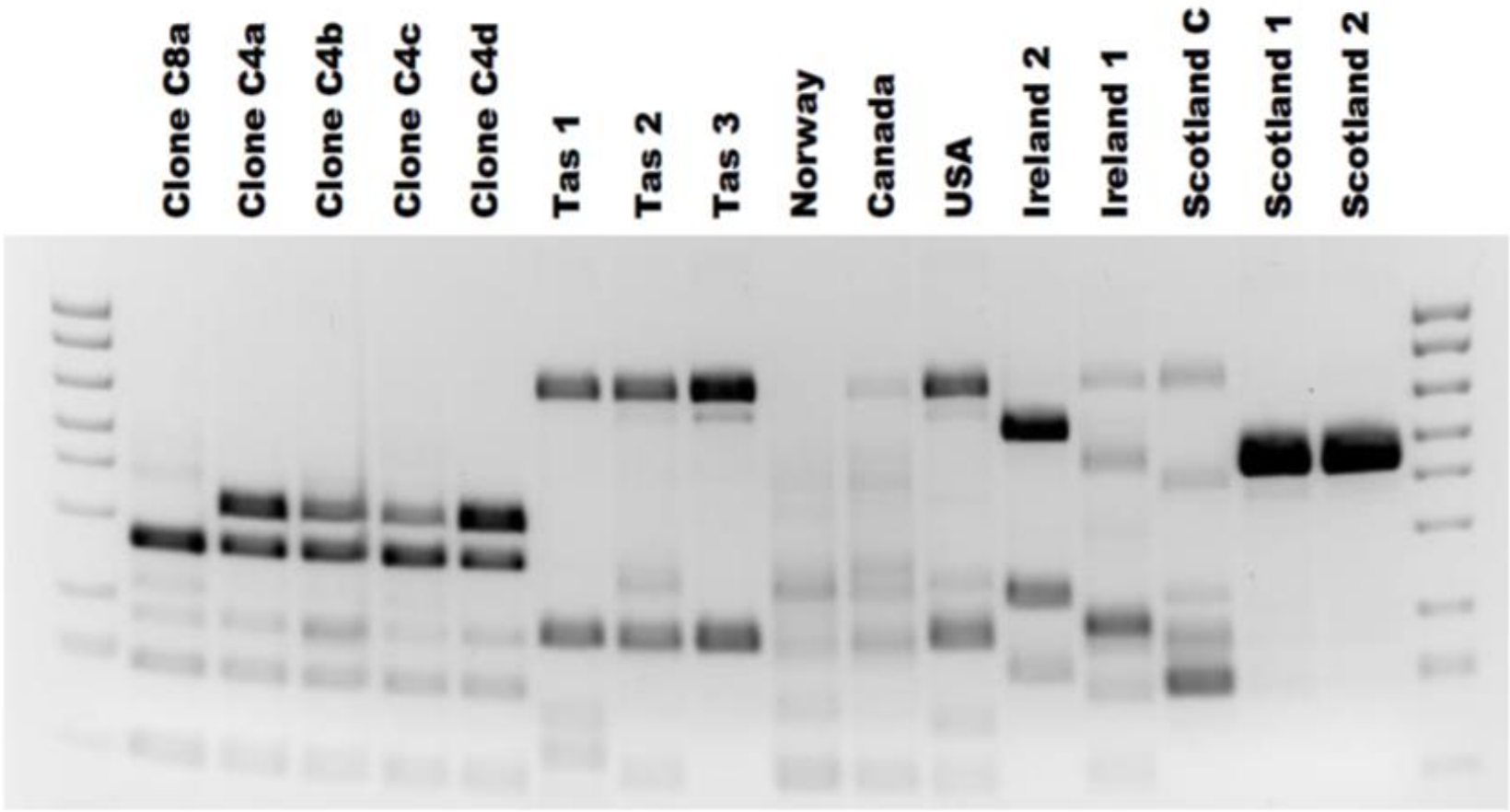

Figure 3.12 Agarose gel of amplified RAPD products for primers B18. 1, Clone C8a; 2, Clone C4a; 3, Clone C4b; 4, Clone C4c; 5, Clone C4d; 6, Tas 1; 7, Tas 2; 8, Tas 3; 9, Norway; 10, Canada; 11, USA; 12, Ireland2; 13, Ireland1; 14, Scotland clone; 15, Scotland 1; 16, Scotland 2.

3.4 DISCUSSION

Sixteen samples of *N. perurans* were compared based on RAPD analysis to determine genetic relatedness at the sub species level. The data presented in this study suggest that *N. perurans* genomes are far more polymorphic than the MLST data would have suggested. Each individual primer showed a different pattern and in turn produced unique phylogeographic groupings. Each primer showing a different banding pattern is not unexpected considering that the primers consist of different combinations of base pairs meant to attach at different location along the genome (116). The study on *Naegleria fowleri*, the source of the primers used in this study, shows this same type of five primer/ five profiles pattern (156). Similar to this study, the *Naegleria* isolates showed pattern diversity within any one given primer (156). Dendrograms were not made for each primer in the *Naegleria* study so a direct comparison of the changes in groupings each primers predicted is not possible (156). Similar to *N. perurans*, the RAPD analysis found that there was greater variation within isolates from specific countries (156). In *N. perurans* there appeared to be the greatest sequence similarity amongst the Tasmania samples, specifically the clonal samples. This is not surprising given that the majority of the samples surveyed came from Tasmania. Similar consistency may be observed in other locations with the addition of more samples. It should be noted, that large band number per primer and banding patterns with similar discrepancies are also seen in other RAPD studies. The banding patterns produced for *Entamoeba histolytica* show similar patterns when comparing pathogenic and non-pathogenic samples (157, 158). These studies however had a larger proportion of conserved bands across the multiple samples of each type compared and thus showed better resolution and higher levels of discrimination (157, 158).

In this study, there were few conserved bands across all the samples for any given primer. This apparent higher level of variant banding could be due to length heterogeneity between samples within the genome. These length heterogeneities could provide additional sites for the non-determinate primers to bind (69, 80). There is evidence that length heterogeneity occurs in the ITS regions of *N. perurans* sister species *N. pemaquidensis* and *N. invadens* (62, 80). Since the primers are arbitrary set of base pairs, the RAPD profiles potentially contain fragments from the Parasomes genetic material as well. Regardless, as far as we know, *Neoparamoeba* reproduces asexually with the parasome passed on from mother to daughter cells and thus should follow the same selections pressures. Without further investigation, it is impossible to tell how much the banding patters are affected by the parasomes genetic material. When combined, the resulting dendrogram has some points in agreement with that of the MLST analysis following similar geographic patterns. There is more resolution in the RAPD analysis which gives insight to the potential relationships. Take the Australian samples for instance. In the RAPD analysis, the Tasmanian sample group seen in the MLST analysis has been split. RAPD now groups the wild samples all together and the clonal samples altogether but separate from each other. This would indicate that there are genetic differences between the clonal and wild isolates that could not be determined with MLST. This is an observation we would expect given that changes in virulence between clonal and wild *N. perurans* have already been observed and likely have a genetic basis (141).

This study contains a larger geographic range comparison than seen in most RAPD literature dealing with parasites. In addition, there are very few samples per location, with the exception of Australia, which could influence the dendrogram organization. When the

marine nature of *N. perurans* is considered, the lack of strong distinctions between each geographic location is further explained. Marine organisms face fewer constriction to their dispersal and thus are less likely to face barriers in gene flow (15). For instance the marine bacterium, *Vibro vulnificus,* also displays a similarly diverse genomic arrangement when RAPD analysis is applied to samples from hosts in the environment (167). *N. invadens,* the disease agent for mass mortality in green sea urchins, has been found to be temperature sensitive and unable to permanently reside in waters along the east coast of Canada (153, 168). Rather, the continued mass mortality events appear to be linked to storm events where warmer waters were introduced into the area (153). Temperature also appears to play a role in the biology of *N. perurans* and the risk of AGD outbreaks (42). Similar situations to *N. invadens* could potentially occur in *N. perurans* where storms or sudden changes in temperature allow for an influx of amoebae into new areas or local populations.

In addition to its usefulness with genetic fingerprinting, based on our results, RAPD appears to have some practicality in regard to virulent subtypes. Within our analysis were included clonal isolates that have been kept in culture for three years and shown to be avirulent after passage (141). They showed a different pattern to wild samples obtained from the same location, which was particularly apparent in the banding patterns of primers, B10, B12 and B18. The differences in patterns may be indicative of genetic changes in line with losing virulence. Previous studies using RAPD in parasitic or pathogenic eukaryotic species were shown to be useful in differentiating between highly virulent, less virulent and avirulent strains (169, 170). In particular, RAPD was useful for the crayfish fungal pathogen *Aphanomyces astaci,* where it was used first to differentiate between five genotypes based

primarily on geography and then to show new outbreaks were caused by a genotype not previously described (170).

Though, there is some ambiguity present in the results of the both the MLST and RAPD analysis for *N. perurans,* a combination of these analysis would still be preferred over the addition of a new typing, method such as RFLP. As stated in the introduction, the length heterogeneity interfered with the RFLP analysis (80). This length heterogeneity appears to be random and even clonal cultures can have differences from one generation to the next (62, 80). This makes it an unreliable marker, not to mention that the majority of samples that would be compared in future analysis would be mixed as it would not be feasible to make clonal cultures of every sample prior to analysis. In addition, the similarities between MLST and RAPD are promising and indicate that refining these analyses would prove beneficial for typing *N. perurans.*

Notably, the B10 RAPD primer dendrogram showed grouping patterns that fit the predicted relationships seen in the MLST analysis. This would suggest that the primer B10 could be used to rapidly predict where new samples best fit within geographical genotype populations of *N. perurans*. This is similar to the *Naegleria* study, which also found that some samples could only be differentiated using the B10 primer (159). These results also indicate that separate evolution at geographic locations is occurring, which in turn would indicate the potential for local population. If this is the case, then the question remains as to where the local source populations are located. Identifying the local source population could help our understanding of the epidemiology of the disease and potentially help identify differences in pathogenicity, as we know that there is potential for avirulence in *N. perurans* (141). Further work on investigating primer sets to resolve a more robust RAPD analysis for *N. perurans* should also be considered as it has proven useful in species- level

differentiation within *Acanthamoeba* (135, 171, 172). In conclusion, RAPD could be a useful tool in *N. perurans* population epidemiology studies. The method is quicker and more cost efficient than MLST and differentiates better between geographic samples than MLST making it a valid option as a first point in identifying outbreak source locations.

Conclusion

This study developed an additional typing method for *N. perurans*. The method is quicker and more cost effective than MLST. It has the added benefit of differentiating deeper between geographic samples making it a valid option as a first point in identifying outbreak source locations. In addition, the observable changes between the clonal samples indicates that RAPD has the potential to be a valuable tool in identifying genetic changes occurring between amoeba clones. This could be particularly useful in virulence studies such as between avirulent and virulent amoebae clones.

In addition to the analytical usefulness of RAPD, in this study the breadth of the heterogeneity between samples observed suggests that there are localized populations that are changing independently of the 'global' population. This is significant as it would indicate that there may be reservoir populations which have the potential to infect and re-infect farm sites. Given that we know from the MLST research that outbreaks are environmentally driven, identifying potential reservoirs is even more crucial for AGD research.

CHAPTER FOUR: DETECTION OF *Neoparamoeba perurans* IN SEDIMENT

4.1 INTRODUCTION

Neoparamoeba perurans is a ubiquitous organism and genotypic differences have been reported for samples from separate geographic locations, which suggests that there may be reservoir populations of *N. perurans* (Chapter 2 and 3). Understanding if and where an environmental reservoir occurs improves our understanding of the disease contraction and spread (173). Further, understanding the potential risks can help in the design and implementation of management strategies (173-175). In the southern bluefin tuna (*Thunnus maccoyii*), ranching industry at Port Lincoln in South Australia, moving and maintaining the fish at an offshore location as opposed to the normal inshore Tuna Farming Zone (TFZ), resulted in better health and reduced obligate parasite load (176). Distance from both the shore and waterbed were considerable risk factors for parasite (*Cichlidogyrus* sp., *Bolbophorus* sp., *Acanthogyrus tilapiae* and *A. macracantha*) infections in Nile tilapia (*Oreochromis niloticus*) farmed in Uganda (173). These findings demonstrate the importance of identifying pathogen reservoirs, such as surrounding water because tidal fluctuations created a dispersion pathway for parasites (173, 175, 177, 178). In addition, high levels of infectious parasite stages within a reservoir increases the risk of infection, especially when coupled with high host densities (152, 177). The ability to detect these pathogens in the environment is a key to risk assessment and mitigation in farming site selection (174).

Historically sediment has been the most challenging and least well surveyed of the potential reservoirs for pathogens (Chapter 1, (15, 16)). There is, however, evidence that

Neoparamoeba and *Paramoeba* reside within benthic sediments (63, 82, 83, 89). *Paramoeba atlantica* and *Neoparamoeba aestuarina* have been isolated from samples taken from bottom sediment in the Atlantic Ocean and *Neoparamoeba longipodia* from sediments originating from the Sea of Japan (63, 89). In addition, *Neoparamoeba* sp. has been cultured from the sediments around Tasmania (82, 83). Originally it was thought that the detected species was *Neoparamoeba pemaquidensis* (82), however, this was amended to *Neoparamoeba* sp., as it was later found that the PCR primers were nonspecific and may have falsely identified *Neoparamoeba branchiphila* as *N. pemaquidensis* (82, 83).

These previous studies identify the presence of *Neoparamoeba* and *Paramoeba* in sampled sediment, using a variety of methods, each with its own advantages and disadvantages. The results of those studies depended not only on the detection method (culture or direct PCR) but also on sampling method (See table 1.8). One of the more common sediment sampling methods is the use of a grab (primarily Van Veen Grab) sediment sampler (Figure 4.2) (46, 63, 83, 118). The grab is a gravity sampler that comprises a large set of jaws that are held open arms and a hook latch (179, 180). The sampler is then lowered from the surface using a winch line and the release mechanism is activated when jaws make contact with the sediment (179, 181). This relieves pressure on the latch and the jaw clamps shut collecting a large amount of sediment which is then reeled back to the surface (179, 181). The grab sampler is most effective in softer sediments but the device does have a tendency to lose fine material or fail to close properly due to gravel caught in the jaw (179, 181).

Van Veen grab systems were used in the sediment surveys for *Neoparamoeba* done around Tasmania in 2005 (83), and in the most recent survey done in Norway (118). The 250cm^2 Van Veen grab system has the capacity to collect a little over three litre samples; however only portions of the top layer were used (59, 82, 118, 180). In the earlier sediment sampling of Tasmania, two hundred grams to eight hundred grams of the sediment collected by the grab bucket at each site were taken but only three to five grams were used per sample in an attempt to culture the amoebae (82, 83). In the most recent study in Norway the full amount at each location was collected but only between two and ten, two cubic millimetre samples were processed (118). *Neoparamoeba* sp. were detected in Tasmanian sediments but not in the Norwegian sediments, however the detection method in Tasmania was based on culture enrichment PCR whereas in Norway PCR only was used which may have incurred inhibitors from the sediment or may not have been sensitive enough to detect small numbers (82, 83, 118).

Other commonly used techniques are the collection of sediment manually using either divers or a Remotely Operated Vehicles or ROVs (Figure 4.6) (46, 182). Divers are advantageous as it is easier to sample exactly where and what is intended without worrying about disturbing the surrounding sediment (183). The 2010 study along the western coast of the United States of America and Canada, used both divers and grab systems (Petite-Ponar) to collect two mL of sediment for processing (46). Remotely Operated Vehicles are similarly advantageous, however the sample size that they can collect is limited and their usefulness has yet to be tested for *N. perurans* sediment surveys (183).

Molecular species identification has become the standard due to the difficulty of microscopic species identification of marine amoeba and the inconsistencies and imprecision of other common differentiation methods such as IFAT (45, 59, 67). Extraction of large amounts of high quality DNA from sediments however, can be difficult (184). Extraction of DNA from sediment for the purposes of this study can be separated into two components: 1) processing sediment for the removal of cells and 2) extraction of DNA. Previous studies have approached processing of sediment in a variety of way. Crosbie et al. (2003, 2005) allowed the amoebae to migrate from the sediment by inoculating MYS plates with two to five grams of sediment and incubating the plates therefore removing the potential of sediment inhibition (82, 83). Sub-culturing on the MYS plates was then done and incubated again until there were enough amoebae to run IFAT and proceed with DNA extraction and PCR analysis (82, 83). This method was effective with positive identification of *Neoparamoeba* sp. occurring in both studies over multiple test locations (82, 83). These studies however, were done prior to *N. perurans* being identified as the causative agent of AGD in 2012 and because they relied on a number of culturing steps are not a suitable indication of amoeba abundance in the sediment at the time of sampling (44, 55, 82, 83).

The next sediment census along the western coast of the USA and Canada, preserved the sediment immediately after sampling (46) with 2 mL per sample fixed with 10 mL of ninety-five percent ethanol (46). Once a precipitate formed in the ethanol above the sediment sample, 1 mL of that was removed and DNA was extracted using a plant DNA isolation kit from MoBio (46). Soils and sediments often contain high number of PCR inhibitors such as humic acid (184). The high organics content of the benthic sediments appears to contribute to accumulation of inhibitors in a sample over time making quick

processing of samples important for increasing sensitivity (184). As in the previous study, the most common method for DNA extraction is commercial DNA or RNA extraction kits (46, 118). These kits contain patented solutions as part of their extraction protocol which remove as much inhibitory material as possible during the process. The kits however tend to have reduced DNA yield and less clean DNA compared to traditional isopropanol extractions (185). In addition to reduced yield kits can only process a relatively small sample size (3-5 g), especially when it is considered that the benthic sediment surface area is 2246m^2 under an average 20,106 m^2 salmon pen (186).

In the study in Norway, two mm^3 of sediment were taken, kept on ice and incubated in 750 mL of QIAzol, a lysis reagent used in RNA purification (118). Following the incubation, RNA was purified using a Qiagen kit prior to qPCR (118). Similar to the study in western North America, small volumes of samples were used with a limited number of samples per site, and RNA, rather than DNA was extracted (46, 118).

Two primary areas where current nucleic acid extraction techniques from sediment can be improved are sample volume and reduction of the effect of PCR inhibitors. In order for a DNA extraction method to be useful in the context of sediment screening, methods need to be developed that are economic (i.e. minimized labour, costs, supplies), and produce high DNA yields with little DNA degradation and minimal PCR inhibition (187).

Here, two methods were tested for the extraction and quantification of *N. perurans* DNA from marine sediments. The first method sacrifices some sensitivity and potentially incurs the effects of inhibitors but allows for long-term sample storage. This method was applied to samples from the coast of Vancouver Island, British Columbia, Canada. The

second method focused on sensitivity, using a differential centrifugation technique that allows for the removal of cells without accumulating additional inhibitors, but requires processing of the samples within 48 hours. This method was applied to samples from Hideaway Bay, Tasmania. This thesis does not compare those methods rather examines two distinct methods for the detection of protozoan pathogens in sediment each with potential advantages dependant on circumstance.

4.2 MATERIALS AND METHODS

4.2.1 PRESERVATION METHOD FOR DNA EXTRACTION FROM SEDIMENT – BRITISH COLUMBIA CASE STUDY

4.2.1A SAMPLING

Sediment samples were collected from eleven Atlantic salmon farm leases along the coast of Vancouver Island, six were along the northern coast (Bull Harbour, Bell Island, Midsummer Island, Doctor Islet, Althorpe Point and Okisollo Channel) and four along the western coast (Koskimo Bay, Monday Rocks, Mahatta West, Mahatta East and Cleagh Creek) (Figure 4.1). The sampling sites were chosen and carried out by Marine Harvest, Canada in accordance with the Department of Fisheries and Oceans (DFO) guidelines for benthic monitoring (Figure 4.3). The conditions across the sites ranged from fallowed (not stocked) for over 2 years to actively stocked with no recent fallowing period (Table 4.1). At each site,

three sediment samples were from each sampled point; a reference point and three points at a different distance from the seacage (0 meters, 15 meters and 30 meters) diagonally out from the edge of the seacages using a Van Veen grab bucket sampler (Figure 4.2). In addition, Marine Harvest, Canada, provided sediment analysis data for each of the sites (Table 4.2). The data provided were for samples collected at the 30 m sampling point and included the total volatile solids (TVS), percentage and sediment grain size (SGS), percentage of gravel, sand and mud (188).

Once the sediment was brought up by the Van Veen grab bucket, between 30 and 50 g was aliquoted into sample containers on site and kept on ice during transport to the lab. The samples were stored at -80°C for over four months prior to processing. The samples were then removed and thawed overnight at 4°C. Once thawed, between 20 and 21 g (approximately 15 mL) of each sample were transferred into a sterile 50 mL tube for further processing. The remaining sample was returned to -80°C for storage.

4.2.1B SAMPLE PROCESSING

The samples were processed by first adding 35 mL of 1% sodium dodecyl sulfate (SDS) to the tubes containing 15 mL of sediment, then inverted by hand to suspend the sediment before being placed on a Barnstead Mini MaxQ 4450 Orbital Shaker/ Incubator (Hyland Scientific, Washington, USA) set to 23°C for 1 h. Incubation on the shaker was critical to homogenize the sample and allow time for the cells to lyse efficiently. After incubation the tubes were removed and centrifuged at 5000xg for 10 min the supernatant

was then decanted to a sterile 50 mL tube and 500 µL was transferred via pipette to a 1.7 mL tube and the remaining supernatant was stored at -30°C.

Figure 4.1 Sampling sites along the coast of Vancouver Island, British Columbia. 1, Bull Harbour; 2, Bell Island; 3, Koskimo Bay; 4, Monday Rocks; 5, Mahatta West; 6, Mahatta East; 7, Cleagh Creek; 8, Midsummer Island; 9, Doctor Islet; 10, Althorpe Point; 11 Okisollo Channel.

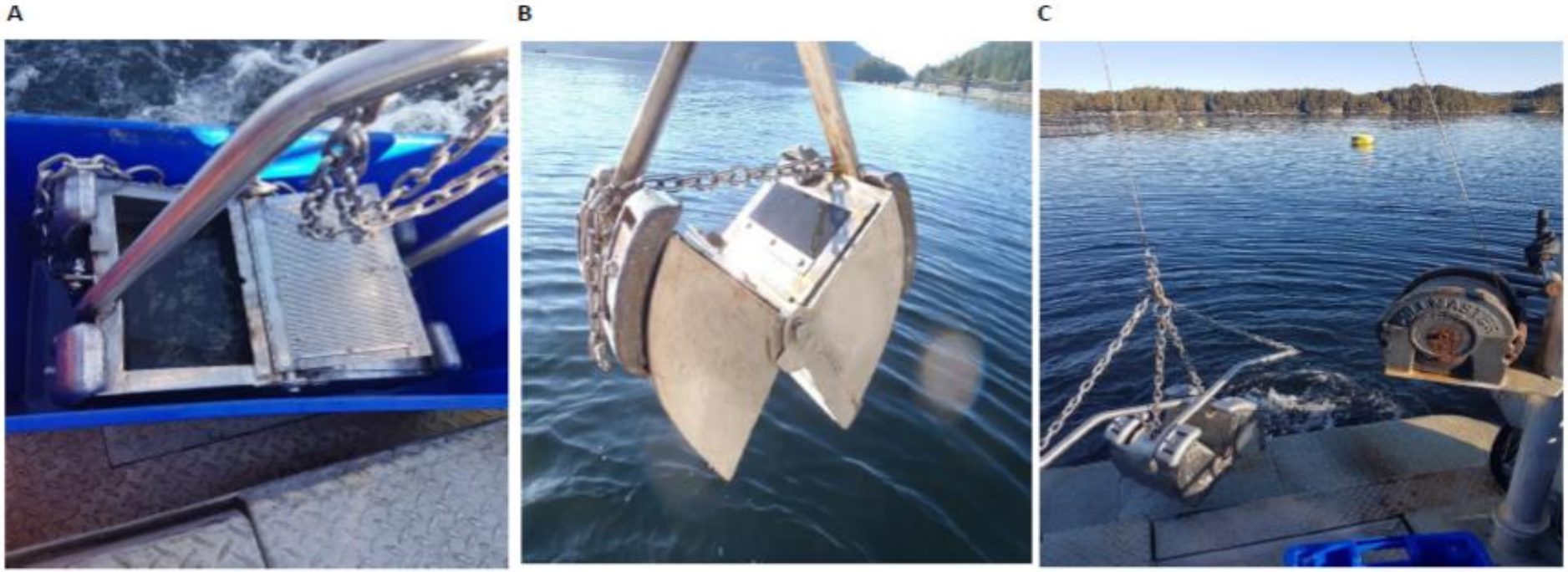

Figure 4.2 Images of the Van Veen grab bucket system used to sample sediments from the coast of Vancouver Island, Canada. A) The system in relation to the vessel, prior to deployment, with the winch system visible; B) Close up of the sampling bucket; C) the Van Veen grab is lowered into a container to catch the sediment collected. The samples used in the study were taken from the top of the sediment deposited in this container.

Figure 4.3 Schematic of sampling locations relative to the commercial seacages. Marine Harvest, Canada, use rectangular pens adjacent to the shore for raising salmon. Samples were taken at a distance of 0 m, 15 m, and 30 m diagonally out from the edge of the seacage.

4.2.1C DNA EXTRACTION

An isopropanol precipitation was carried out on 500 µL of the supernatant from each sample. After a 5 min centrifuge at 16,000×g, the supernatant was decanted into a sterile 1.7 mL tube then 250 µL ammonium acetate (7.5M) were added to the supernatant and vortexed for 20 s prior to centrifugation at 14,000×g for 5 min at 18°C to remove undigested protein. The supernatant was decanted into a sterile 1.7 mL tube and placed at 37°C for 5 min prior to precipitation to ensure that any remaining SDS dissolved into solution. Equal volumes of isopropanol with co-pink (Bio-line, USA) were added; the tubes were inverted 40 times to mix and left on the bench for 30 min to ensure optimal nucleic acid recovery. The samples were centrifuged at 16,000×g for 30 min to pellet the nucleic acids, and the pellet was washed 2 times with 70% Ethanol. The samples were centrifuged for 10 – 15 s, and all the residual ethanol was removed. The pellet was suspended in 50 µL elution buffer and stored at -30°C.

4.2.1D QPCR ASSAY FOR *Neoparamoeba perurans*

Real-time PCR assays were performed using the primers QNperF3 and QNperR3 (68) and the HEX (6-Carboxyhexaflourescein)-labelled and Black Hole Quencher 1 (BHQ1) quenched probe (119). The reactions were run using a Quant Studio 12flex (Applied Biosystems, CA, USA). Each 10 µL reaction consisted of QNperF3 and QNperR3 primers at a final concentration of 600 nM each, a QNperProb at 200 nM, 2 µL of template DNA and 2x MyTaq HS DNA polymerase mastermix (Bioline, USA). The PCR protocol was as follows: an

initial step of 95°C for 3 min, followed by 95°C for 10 s and 55°C for 30 s repeated for 40 cycles. All samples were initially run in duplicates.

4.2.1E LIMIT OF DETECTION FOR DNA PRESERVATION METHOD

Real-time PCR performance was evaluated according to the lower limit of detection (LOD). The LOD is the smallest amount of template DNA that can be reliably detected and amplified in 90% of replicates (189). The LOD for the DNA preservation method was determined using real-time PCR on spiked sediment samples from a reference site along the coast of Vancouver Island. Amoebae used for spiking were isolated at BC CAHS from AGD infected salmon collected from Marine Harvest farm sites. Prior to spiking, a subsample of the sediment was processed as above to ensure there was no detection of *N. perurans* DNA in the sediment used for spiking experiments. Six independent 20 g samples of sediment were spiked with a range of amoebae (1000 cells, 500 cells, 250 cells, 100 cells and 10 cells) with the final sediment sample as a negative control. The first four quantities, 1000, 500, 250, 100 cells, were counted and calculated using a haemocytometer. The last quantity, 10 amoebae, were single cell picked and counted manually to ensure accurate numbers. The amoebae were added to the sediment prior to the addition of the SDS and the DNA extraction protocol was followed as above. Ten PCR replicates of each sample were run using the PCR protocol above and the LOD was set at the lowest cell quantity that returned a minimum of 9 amplifications out of the 10 replicates or 90%.

4.2.1F QUALIFICATION OF POSITIVE IDENTIFICATION OF *Neoparamoeba perurans* DNA

The amoebae number can be calculated using the output from the CFX Connect PCR Detection System utilized in the IMAS Molecular Lab and calculations using the program R (68). For the British Columbia samples, amoebae numbers could not be calculated due to the differences in output from the Quant Studio PCR machines used by the B.C. Centre for Aquatic Health Sciences (CAHS) were the samples were processed. Therefore, a sample was determined to be positive for the presence of *N. perurans* DNA when amplification was observed in duplicate replicates and absent when no amplification occurred. Samples that had amplification in one of the two replicates were repeated in a 10-replicate run and were considered a weak positive when a minimum of six of the ten replicates amplified DNA.

4.2.2 DIFFERENTIAL CENTRIFUGATION METHOD FOR DNA EXTRACTION FROM SEDIMENTS – TASMANIAN CASE STUDY

4.2.2A SAMPLING

Sediment samples were collected from three sites around the coast of Tasmania (Figure 4.4). Three samples were collected by a diver at the first two sites (sites 1 & 2, Figure 4.4). These six samples were opportunistically collected and were not able to be transported for processing immediately therefore they were preserved using the DNA preservation method. At the remaining site (site 3, Figure 4.4), samples were collected from under five commercial Atlantic salmon seacages at two locations within one farming lease.

In addition, one location upstream of the salmon seacages was sampled as a control (Figure 4.5). All sampling locations within site 3 were located in the Huon Estuary, eastern Tasmania (site 3, Figure 4.4). All seacages sampled have been actively stocked and rotated since 2016 with no recent fallowing with the exception of the reference location, which has never been farmed. At each salmon seacage, three sediment samples were taken from three separate points under the cage at a depth of 30m. Samples were taken using a VideoRay Remotely Operated Inspection system (Pro 4 model) that had a manipulator arm and sediment sampling attachment (Figure 4.6). The precise location of each spot under the cage is not known as no GPS data were recorded. The sediment was transferred from the sampling arm into sterile 50 mL tubes up to the 15mL mark (each sample weighed between 20 and 21 grams) and stored in a cooler while on the vessel, prior to being placed on ice for transport back to lab. Once at the lab, the samples were transferred to a refrigeration unit and processed within 48 hr of collection

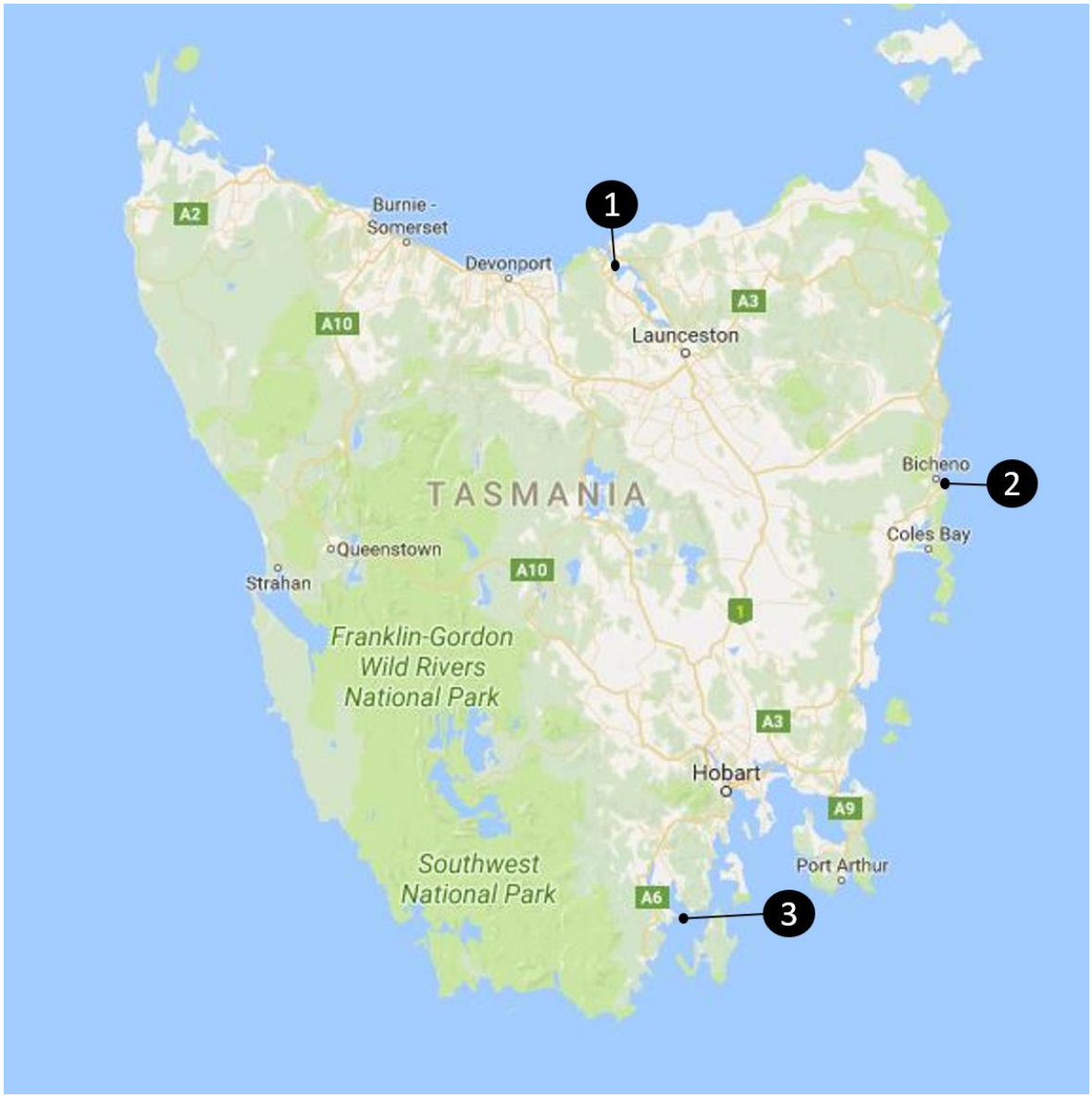

Figure 4.4 Sampling sites along the coast of Tasmania, Australia. 1, Tamar Estuary near George Town; 2, Waubs Bay near Bicheno; 3, Hideaway Bay near Dover. Three samples were taken from site 1 and 2 by a diver, and eighteen samples (3 per location within the site) were taken from site 3 via ROV.

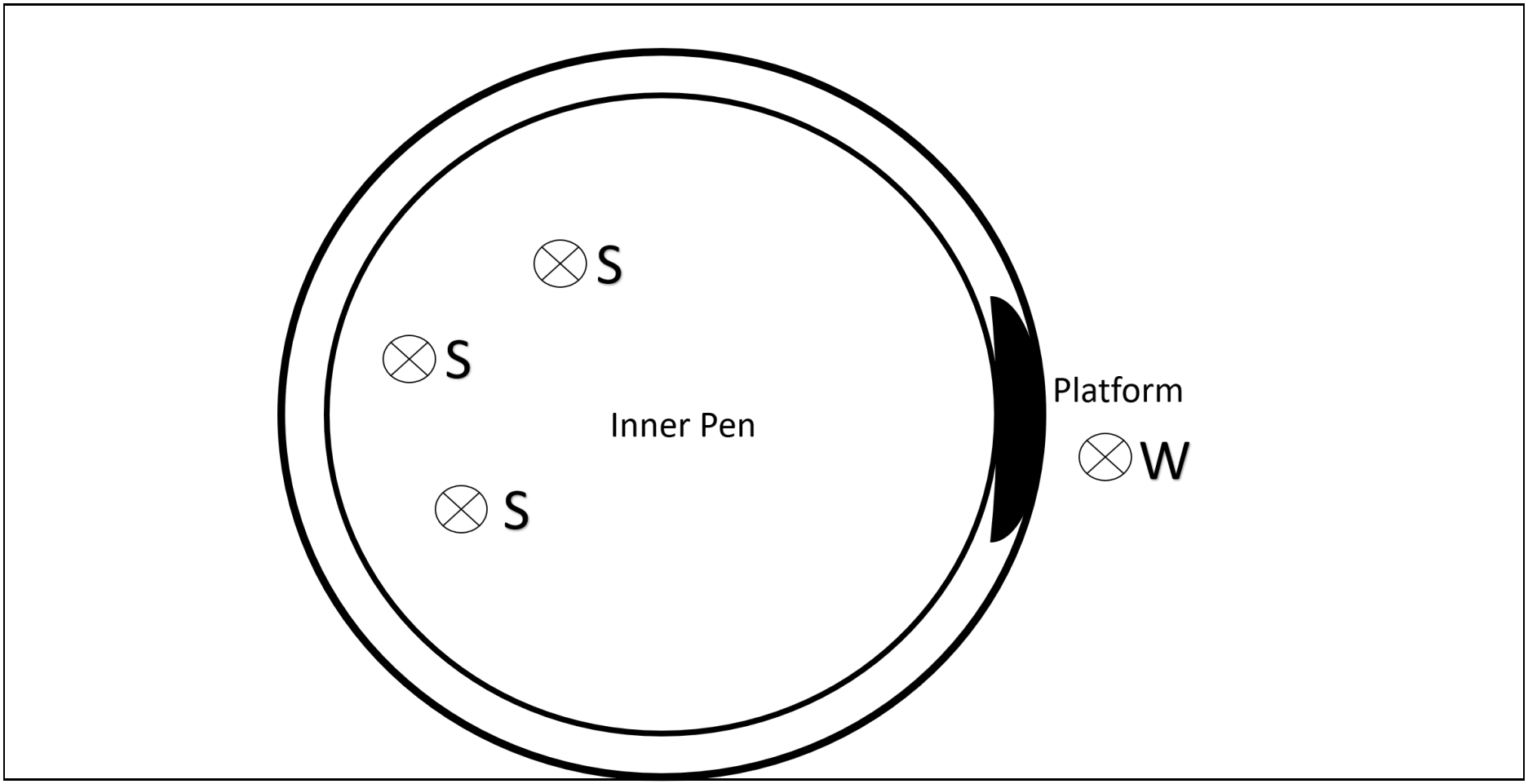

Figure 4.5 Schematic of approximate sampling locations relative to commercial pens. Circular pens are used at Tasmanian sites where sediments samples (S) were taken using an ROV at locations under the pens. The water samples (W) were taken using a Nisken bottle near the platform at the edge of the cage.

Figure 4.6 Image of the VideoRay Remotely Operated Inspection system (Pro 4 model) with a manipulator arm used to sample under salmon seacages at Tasmanian sites.

4.2.2B SAMPLE PROCESSING

The samples from site 3 were processed by adding filtered seawater to the tubes containing the 15 mL of sediment, which were gently shaken manually to homogenize. The tubes were then centrifuged at 50×g for 5 min. The supernatant was collected in a sterile 50 mL tube and centrifuged for an additional 5 min at 800×g to pellet the cells. The supernatant was then removed and returned to the original tube containing sediment, leaving a pellet. 3 mL lysis buffer (4 M urea, 1% SDS, 0.2 M NaCl and 1 mM sodium citrate) was added to the tube containing the pellet. The procedure was then repeated on the original sediment sample to produce a second pellet. The lysis buffer containing the initial pellet was added to

the tube containing the new pellet, thus both pellets were re-suspended in the same 3 mL of lysis buffer.

4.2.2C DNA EXTRACTION

The DNA extraction for the samples from site 1 and 2 was done as reported for the British Columbia samples. The DNA extraction for the samples from site 3 was similar to the one reported for the DNA preservation method above with a few changes. Briefly, for the sediment samples, the final pellet was washed 2 times with 60% Ethanol instead of 70% prior to pellet resuspension. The change in ethanol concentration was due to a recent study that outlined DNA folds faster and is more stable in 60% than in 70% co-solvent (190). The samples then underwent an additional spin column clean-up, which was as follows: 50 µL of binding buffer (3M GuHCl 3.75M NH4Ac pH 6) was added to the 50 µL of elution buffer then 50 µL of 100% ethanol was added to the sample before it was immediately transferred to a spin column. The sample was centrifuged at 10,000×g for 1 min. The flow-through was discarded and 500 µL of washing buffer was added followed by a 1 min centrifuge at 10,000×g. This step was repeated a second time before the column was centrifuged for 2 min at 10,000×g to remove all remaining ethanol. The column was placed into a sterile 1.7 mL tube and 40 µL of pre-warmed (55°C) elution buffer was added to the column and left to incubate for 1 min before being centrifuged at 10,000×g for 1 min. This step was repeated using the same 1.7 mL tube for a final volume of 80 µL.

4.2.2D QPCR ASSAY FOR *Neoparamoeba perurans*

Real-time PCR assays were performed using the primers QNperF3 and QNperR3 (68) and the HEX (6-Carboxyhexaflourescein)-labelled and Black Hole Quencher 1 (BHQ1) quenched probe (119). The samples were run using a CFX Connect Detection System (Bio-Rad, NSW, Australia). Each 20 µL reaction consisted of QNperF3 and QNperR3 primes at a final concentration of 400 nM each, a QNperProb at 100 nM, 4 µL of template DNA and 2x MyTaq HS DNA polymerase master mix (Bioline, USA). The PCR protocol was as follows: An initial step of 95°C for 3 min, followed by 95°C for 10 s and 55°C for 30 s repeated for 45 cycles. All samples were run in duplicates.

4.2.2E LIMIT OF DETECTION AND LIMIT OF QUANTIFICATION FOR CENTRIFUGATION METHOD

The LOD for the differential centrifugation method was determined using real-time PCR on DNA from six independent 20 g samples spiked with a range of amoebae (1000, 500, 100, 10 and 1) that had been counted using a heamocytometer, and manually for amounts smaller than 100. Amoebae used for spiking were isolated at the University of Tasmania, from AGD infection salmon propagated in infection tanks at the university. The limit of quantification (LOQ) was calculated for this method. The LOQ can be described as the lowest amplification from replicate samples where all samples have a coefficient of variation (CV) of less than 20%. The LOQ was determined from the LOD samples. The copy number of the 18S gene was determined for the Tasmanian samples following the procedure outlined in supplementary Method S1, Bridle et al. (2015) (141). Briefly, a standard curve was generated using serial dilutions of the manufacturers (Integrated DNA Technologies,

Oregon, USA) double stranded DNA with a portion of *N. perurans* 18S rDNA. Using the standard curve, it is possible to determine the copy number of the gene present in the sample and from this extrapolate the number of amoeba, assuming 2880 copies per amoeba as previously determined (68).

4.2.2F QUALIFICATION OF POSITIVE IDENTIFICATION OF *Neoparamoeba perurans* DNA

As with the DNA preservation method, a sample was determined to be positive for *N. perurans* DNA when amplification was observed in both replicates and absent when no amplification occurred in both replicates. Further, the number of amoebae present in the sample could be calculated using the output data from the CFX Connect PCR Detection System (Bio-Rad). The data were converted to the appropriate format using excel and input into R for the calculation of copy umber against the standard curve as previously described (141). The copy number was then converted to the approximate number of amoebae present using the assumption of 2880 copies per amoebae. In instances where amplification for duplicate samples occurred at different cycles, the samples were re-run using six replicates and the average number from those six replicates was reported for that sample. The means and standard deviation for the number of amoebae present per site was calculated (Table 4.5).

4.2.2G ADDITIONAL ANALYSIS

As reported above the sediment samples received from site 1 and 2 were not able to be processed immediately and were therefore treated similar to the DNA Preservations samples. Fifteen mL of sediment were transferred into a 50 mL bottle and 35 mL of 1%

sodium dodecyl sulfate (SDS) were immediately added to the tubes. The samples were then stored at -20°C once received. The DNA extraction for the samples from site 1 and 2 was done as reported for the British Columbia samples. The samples from site 1 and 2 were removed from -20°C and allowed to thaw overnight at room temperature (approximately 23° C). Once thawed, the samples were inverted by hand to suspend the sediment before being placed on a shaker at room temperature for 1 h and then centrifuged at 5000xg for 10 min. The supernatant was decanted to a sterile 50 mL tube. 500 µL of the supernatant were transferred to a 1.7 mL tube and the remaining supernatant was stored at -20°C.

In addition, water samples were also collected with the sediment samples at site three. Three litres of water were taken at approximately three to four meters depth for each site. The water samples were similarly transferred from the Niskin bottle and into sterile bottles. Both sets of samples were stored in a cooler while on the vessel, prior to being placed on ice for transport back to the lab. All samples were then transferred to a refrigeration unit and processed within 48 hr of collection.

The water samples were processed by vacuum pump filtration. The 3 L from each site were combined and filtered through the same glass microfiber filter (Sigma-Aldrich). The filters were of 1.2 µm pore size and 47 mm diameter. The system consisted of a Nalgene™ filter holder with receiver (Thermoscientific) attached to the laboratory airflow system. The filters were removed from the system by rolling the filter on itself using forceps prior to storage in a 15 mL tube containing 3 mL of lysis buffer (4 M urea, 1% SDS, 0.2 M NaCl and 1 mM sodium citrate). To prevent contamination between sites, the filter holder unit and forceps were washed in a 1:10 dilution of bleach (4.2% sodium hypochlorite) in fresh water.

The DNA extraction for the water samples used the same isopropanol extraction protocol as the previous samples. Prior to DNA extraction, the 15 mL tubes containing the filter and lysis buffer were vortexed to lyse amoeba potentially trapped by the filter paper. 500 µL of the sample were removed and placed in a 1.7 mL tube for the DNA extraction. The final pellet was suspended in 80 µL elution buffer.

4.3 RESULTS

4.3.1 DNA PRESERVATION METHOD – BRITISH COLUMBIA CASE STUDY

A limit of detection of 50 amoebae/g of sediment was determined for the DNA preservation method. This LOD was determined from 1000 amoebae per 20-gram sample equalling 50 amoebae per gram sediment. Only the replicates spiked with a thousand amoebae returned a qPCR amplification. It was therefore determined that the method was not sensitive enough to detect below 1000 amoebae. Six of the eleven sites surveyed (Bull Harbour, Bell Island, Monday Rocks, Mahatta West, Doctor Islet and Okisollo Channel) returned a positive detection of *N. perurans* DNA (Table 4.1). The highest proportion of positive amplification of *N. perurans* DNA occurred at the 0 m sampling point. Five of the nine amplifications observed came from 0 m. Of the remaining four amplifications, one came from a reference site, two came from 15 m and one from 30 m (Table 4.1).

There was no apparent pattern to the condition of the site and the presence of *N. perurans* in the sediment (Table 4.2). Of the six previously or currently fallowed sites, four tested positive for the presence of the amoeba including one that had been fallowed for

sixteen months. However, only three of the four sites that had not been fallowed tested positive. Similarly, whether a site was currently active did not seem to have an effect with only four of the seven active sites testing positive, while two of three non-active sites also tested positive. Even AGD status of a site was found not to be an adequate predictor of the presence of *N. perurans* in the sediment. Only three of seven salmon stocked sites had AGD infections at the time of sampling and of those sediment samples only two were positive. Four sites without AGD tested positive for *N. perurans* in the sediment. Two of those sites were not actively stocked.

Table 4.1 *Neoparamoeba perurans* detection results (numbers positive/ number of samples) per sediment sampling by location for sites from Vancouver Island, British Columbia. N/S denotes no samples collected for that depth

Site	Samples at each site (numbers positive/number of samples)			
	Ref	0 m	15 m	30 m
1 (Bull Harbour)	0/3	1/3	0/3	N/S
2 (Bell Island)	1/3	2/3	1/3	N/S
3 (Koskimo Bay)	N/S	0/3	0/3	0/3
4 (Monday Rocks)	0/3	1/3	0/3	0/3
5 (Mahatta West)	0/3	0/3	0/3	1/3
6 (Mahatta East)	0/3	0/3	0/3	0/3
7 (Cleagh Creek)	0/3	N/S	N/S	N/S
8 (Midsummer Island)	0/3	0/3	0/3	N/S
9 (Doctor Islet)	N/S	1/3	0/3	N/S
10 (Althorpe Point)	0/3	0/3	0/3	N/S

11 (Okisollo Channel)	0/3	0/3	1/3	N/S

Table 4.2 Comparison on the qPCR results for sites from British Columbia with the state of the location at time of sampling including: location, if a fallowed period occurred before the sampling- with the duration in parentheses, if it was active at the time of sampling, AGD status of the location and whether the sediments sampled were positive for *Neoparamoeba perurans* DNA. N/D denotes no data.

Site	Fallowed Period (months)	Currently Active	AGD positive	Sediment Positive
1 (Bull Harbour)	No	Yes	Yes	Yes
2 (Bell Island)	Yes (5)	No	No	Yes
3 (Koskimo Bay)	Yes (4)	Yes	No	No
4 (Monday Rocks)	Yes (4)	Yes	No	Yes
5 (Mahatta West)	Yes (16)	No	No	Yes
6 (Mahatta East)	N/D	N/D	N/D	No
7 (Cleagh Creek)	Yes (24+)	No	No	No
8 (Midsummer Island)	No	Yes	Yes	No
9 (Doctor Islet)	No	Yes	No	Yes
10 (Althorpe Point)	Yes (7)	Yes	No	No
11 (Okisollo Channel)	No	Yes	Yes	Yes

There did not appear to be a clear pattern between the sediment data received from Marine Harvest and the detection of *N. perurans* DNA in sediment (Table 4.3). Across the sites, the total volatile solids percentage ranged from a low of 2.4% to a high of 17.4%. It would be assumed that higher numbers of amoebae would be found with higher percentages of organic solids. Interestingly though, the *N. perurans* positive sites were all within the lowest numbers, 2.4% to 4.4%. The low organic content, however, was not a predictor as *N. perurans* DNA was not detected at other sites with low total volatile solids (site 3 Koskimo Bay and site 7 Cleagh Creek). With respect to the individual sediment particle percentages, *N. perurans* DNA was detected regardless of the gravel, sand or mud percentages.

It is important to consider that the data received was from one sample site (30 m distance) for all locations. This one sample site does not appear to be representative of the sample composition for all the sample sites within a location (reference, 0 m, 15 m, and 30 m). For instance, there were obvious differences in colour and texture observed from the samples at 0 m and 15 m from the Monday Rocks (site 4) location (Figure 4.7). There were also, visible differences in the colour of the collected SDS supernatant, post incubation and centrifugation, from replicate sediment samples as seen at location Mahatta East (site 6) (Figure 4.8). These types of observations were seen across all locations in British Columbia.

Table 4.3 Sediment characteristics at different sites. Data provided by Marine Harvest, Canada; Site, Total Volatile Solids (TVS) percentage, Sediment Grain Size (SGS) gravel, sand and mud percentages at 30 m from the cage edge. Also included is *N. perurans* detection for that location, * denotes positive at the 30 m sampling point.

Site	TVS percentage	SGS percentage			*N. perurans* positive
		Gravel	Sand	Mud	
1 (Bull Harbour)	3	0.7	75.6	23.7	Yes
2 (Bell Island)	3.1	0	82.6	17.4	Yes
3 (Koskimo Bay)	2.9	10.3	61.2	28.5	No
4 (Monday Rocks)	4.4	26.7	45.9	27.3	Yes
5 (Mahatta West)	3.5	1.9	72.8	25.3	Yes*
6 (Mahatta East)	11.1	2.8	59.7	37.6	No
7 (Cleagh Creek)	3.1	0.9	82.8	16.3	No
8 (Midsummer Island)	4.8	0	87.3	12.7	No
9 (Doctor Islet)	2.4	0	88.6	11.4	Yes

10 (Althorpe Point)	17.4	0	80.8	19.2	No
11 (Okisollo Channel)	2.5	N/D	N/D	N/D	Yes

Figure 4.7 Sample received from the Monday Rocks location showing the differences in sediment between sites at one location. The two dark samples (Top) were from the 0m sampling site and the light brown samples (Bottom) from the 15 m sampling site.

A

B

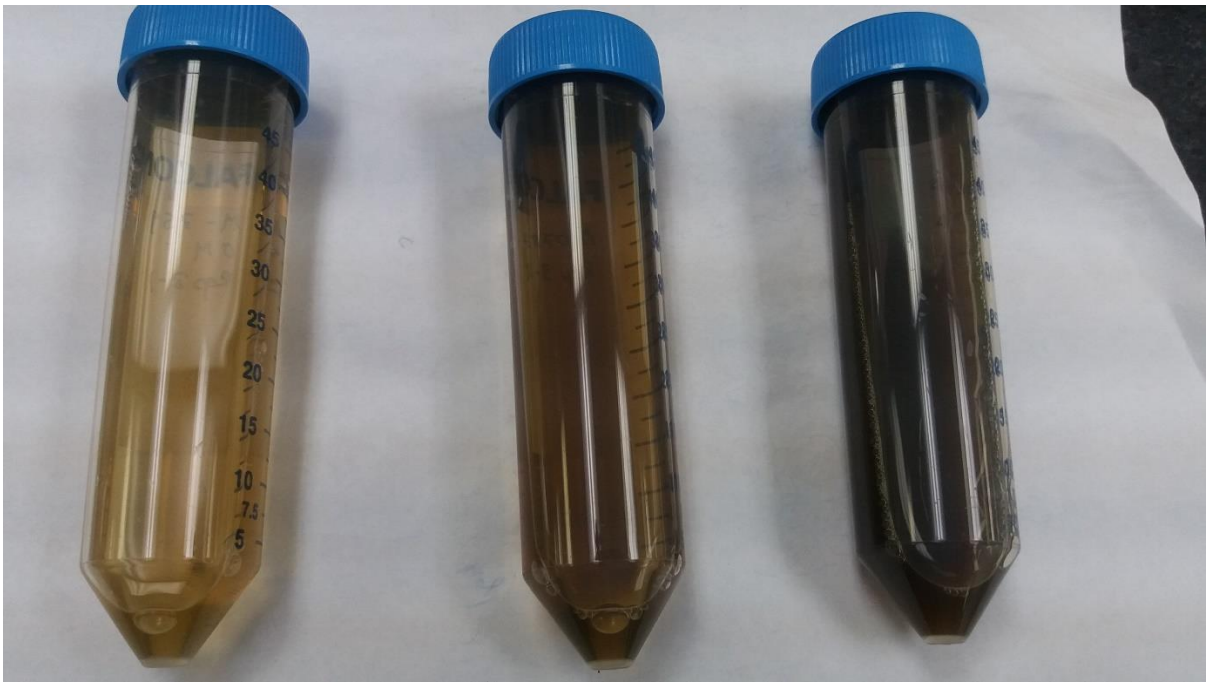

Figure 4.8 A. Sediment replicate samples received from the 15 m sampling site received from the Mahatta East site. B. The corresponding SDS solutions from the samples in A. showing the colour differentiation between the replicates from the same site.

4.3.2 DIFFERENTIAL CENTRIFUGATION METHOD – TASMANIAN CASE STUDY

An LOD of 0.5 amoebae/g of sediment was returned for the differential centrifugation method. The LOQ was much higher at 50 amoebae/g of sediment. All the site 3 samples, except the reference site, returned a positive detection of *N. perurans* DNA. The site 1 and site 2 samples processed using the DNA preservation method were negative (Table 4.4). All of the seacages sampled from site 3 were actively farmed and had current AGD outbreaks. In addition, all the seacages were three and a half weeks post baths except the first location, which was undergoing a bath cycle. The water samples, however, were negative for the presence of *N. perurans* at all but the first, Hideaway bay, despite ongoing infection and bathing at all sites on the eastern coast (Table 4.4). The concentration of amoebae at the positive site was extremely low at less than an amoeba/ litre (0.33/L) (Table 4.4).

Table 4.4 Samples per site and number of amoebae per sample using the qPCR results for locations around the coast of Tasmania. Three samples were taken by diver from site 1 and site 2. No water samples were taken at those location. Three sediment samples were taken by ROV and corresponding water samples were taken with a Niskan bottle from each sample location within site 3.

Site	Sample 1	Sample 2	Sample 3	Water sample Amoeba/ Litre

1 (Tamar Estuary)		0	0	0	N/D
2 (Waubs Bay)		0	0	0	N/D
Site 3 (Hideaway Bay)					
	Location 1	25	0	0	0.33 / L
	Location 2	13	40	0	0
	Location 3	16	21	0	0
	Location 4	40	0	0	0
	Location 5	13	11	4	0
	Control	0	0	0	0

Despite all the sampling locations from site 3 being sediment positive except the control, each location displayed variability in the number of amoebae present per sample (Table 4.4). Location 1, 1/3 samples were positive (25 amoebae); location 2, 2/3 samples were positive (13 and 40 amoebae); location 3, 2/3 samples were positive (16 and 21 amoebae); location 4, 1/3 samples were positive (40 amoebae) and location 5, 3/3 samples were positive (13, 11 and 4 amoebae) (Table 4.4). The status of all of the locations appeared to be uniformly active with the same fallowed status and post bath time with the exception

of site one (Table 4.5). The results indicated variability in the distribution of the amoebae over the sediment under the seacages. There were no data available for the total volatile solids or sediment grain size data available for any of the Tasmanian sites however, the samples appeared more visibly uniform than those collected from Vancouver Island.

Table 4.5 Location details from site 3. Fallowed status; Active status; AGD status; weeks post fresh water bath; sediment status; mean and standard deviation of amoeba per sample collected per site.

Location (cage)	Fallowed	Active	AGD positive	Weeks post Bath	Sediment Replicates Positive	Mean ± SD amoebae per 15ml sample
Cage 1	No	Yes	Yes	0	Yes (1/3)	8.3 ± 14.4
Cage 2	No	Yes	n/a	3.5	Yes (2/3)	11 ± 10.1
Cage 3	No	Yes	Yes	3.5	Yes (2/3)	12.3 ± 11
Cage 4	No	Yes	Yes	3.5	Yes (1/3)	13.3 ± 23.1
Cage 5	No	Yes	Yes	3.5	Yes (3/3)	9.3 ± 2.7
Control	N/A	No	No	n/a	No (0/3)	0

4.4 DISCUSSION

COMPARISON TO PREVIOUS LOCATION SURVEYS

This study has shown the usefulness and sensitivity of two types of sediment extraction methods for the isolation of *N. perurans* DNA from marine sediments associated with Atlantic salmon aquaculture. Each location, however, was assessed with a different method and direct comparisons cannot be made, so each is treated as a separate case study. The British Columbia study occurred first, prior to the development of the centrifugation method that was used in the Tasmanian study. Since the centrifugation method required fresh sediment it was not possible to use this method for the archival samples from British Columbia. The SDS preservation method was applied to samples from site 1 and site 2 from Tasmania but did not return positive results.

A previous study surveyed sediments along the western coast of North America including along the western and northern coasts of Vancouver Island (46) (Figure 4.9). This study was conducted after *N. perurans* was considered the primary aetiological agent and subsequently only the presence of *N. perurans* was targeted (44, 46). In contrast to the present study, there was no detection of *N. perurans* in the sediments surveyed. Only three of the locations sampled in the previous study were in the general vicinity of the locations sampled in this study and none of the sampling locations is identical (Figure 4.10). The difference in the results may be also due to differences in fixation and extraction methods. Here the sample was homogenized prior to a subsample being taken for extraction as opposed to a portion of the precipitate. In addition, the current study used qPCR instead of

PCR. The differences from the previous studies may, however, also be due to a more recent introduction of *N. perurans* to the area. The first reported presence of *N. perurans* on the gills of Atlantic salmon in Canada occurred in 2014 (30). This finding followed a particularly warm winter and a dry summer with limited rainfall (191). It may be that the combination of these two factors created an ideal environment for infection as it did lead to unusual species range shifts in skipjack tuna (*Katsuwonus pelamis*) and the diversion rate of Fraser River sockeye salmon (*Oncorhynchus nerka*) (191).

Similar to the previous two studies, the Tasmanian study found *Neoparamoeba* to be present at farm sites in the Hideaway bay area (82, 83) (Figure 4.10). The other two sampling sites (Tamar Estuary and Waubs Bay) were negative even though *Neoparamoeba* was present in similar locations in 2003 (82). The 2003 and 2005 studies used culturing from sediment samples allowing the amoebae to replicate until visible numbers were present (82, 83). Here, the SDS preservation of the sediment lysed the cells and detection therefore reflected the number of cells present in the sample. This may indicate that *N. perurans* is not present or, since the less sensitive DNA preservation method was used on these samples, they may have been present in too small numbers (less than 50 amoebae/g) to detect. The amoebae numbers returned from site 3 were all below the sensitivity threshold for the DNA preservation method used on site 1 and site 2 so it is not unreasonable to consider this might be the case.

Additional sediments from site 1 and site 2 locations should be examined using the centrifugation method to determine if low amoeba numbers may be present as it is not possible to re-examine the same sediment samples to confirm. Since the previous studies looked for the presence of *N. pemaquidensis* and *N. branchiphila,* it is impossible to say

whether *N. perurans* was present at any of those locations at the time (82, 83). These results, however, indicate that *Neoparamoeba* spp. are present in the sediments around Tasmania. Both studies have shown positive detection of *N. perurans* DNA in environmental sediment samples and both have different potential usefulness for the aquaculture industry but also for the detection of other pathogens.

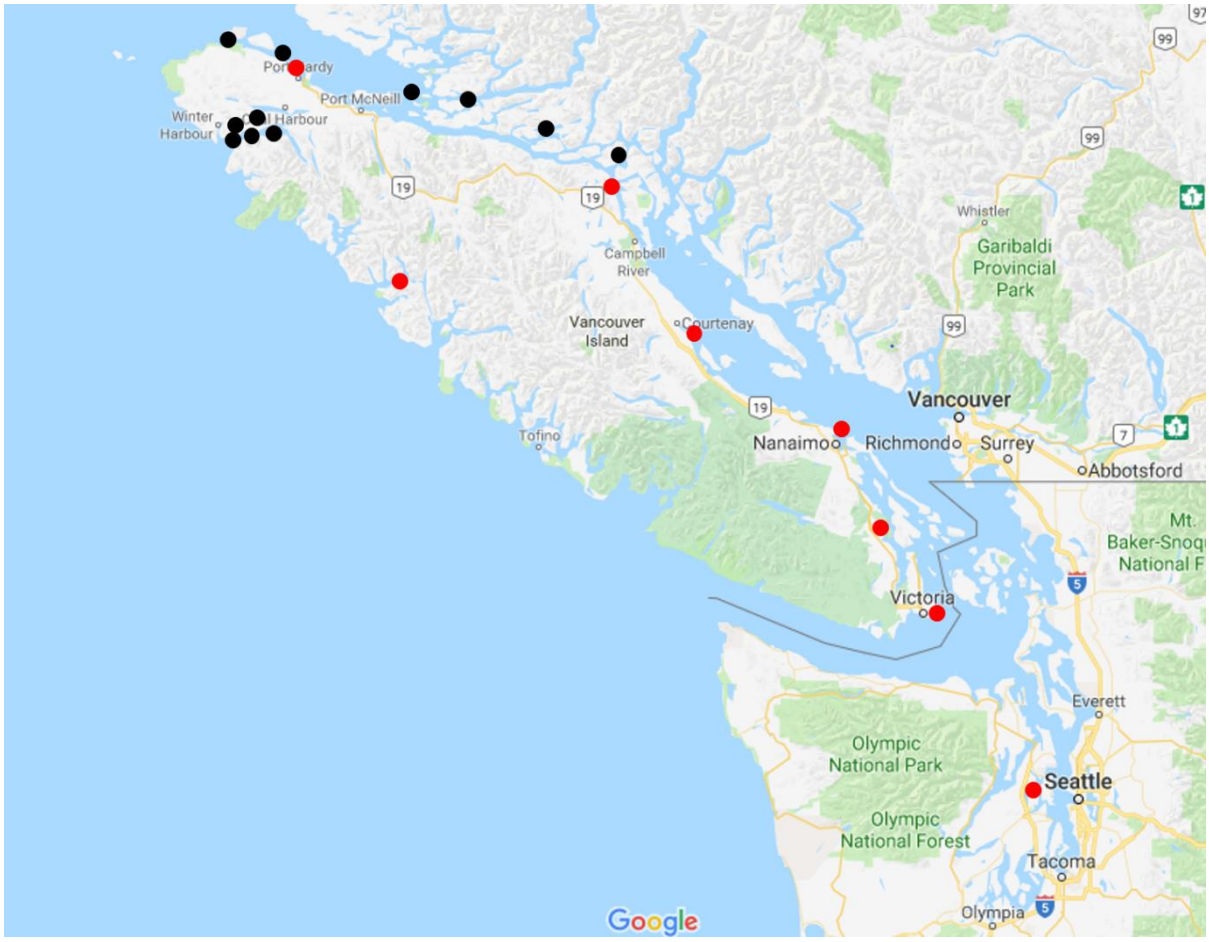

Figure 4.9 Maps of the sampling location from the present study along the coast of Vancouver Island (Black Dots) [Bull Harbour; Bell Island; Koskimo Bay; Monday Rocks; Mahatta West; Mahatta East; Cleagh Creek; Midsummer Island; Doctor Islet; Althorpe Point; Okisollo Channel] and sampling locations taken from Nowak et al. 2010 (Red Dots) [Hurst Island; Tahsis Inlet; Campbell River (commercial farm); Union Bay; Departure Bay (experimental farm); Saanich Inlet; Ogden Point; Puget Sound

Figure 4.10 Maps of the sampling location from the present study (Black Dots) along the coast of Tasmania [Tamar Estuary; Waubs Bay; Eastern Coast near Hideaway Bay] and Sampling Sites surveyed from the Crosbie et al. 2003 study [Stringer's Cove; Nubeena; Hideaway Bay, Tamar Estuary; Tinderbox; Bruny Island; Macquarie Harbour; Bicheno]

COMPARISON OF SDS PRESERVATON AND DIFFERENTIAL CENTRIFUGATION

The use of 1% SDS in the lysis of *N. perurans* cells and preservation DNA within samples appears to be an effective alternative to more costly preservatives such as RNA Later (192). In other studies, SDS has been shown to be equivalent to ethanol and RNA Later in the preservation of marine *Synechococcus* for downstream proteomic studies (193). There was, however, a hundredfold difference in the sensitivity between the two methods reported in this chapter. It is likely that this difference in sensitivity between the SDS preservation method and the Centrifugation method can be attributed to several confounding factors and not necessarily to SDS.

First, the preservation method requires a large volume of SDS in order to fully homogenize and ensure the most efficient lysing of cells in the sample. Attempts were made to extract DNA from the total volume of 35 mL but were unsuccessful due to physical limitation of the tubes during processing especially structural failure during centrifugation. These limitations were unable to be overcome and a 500 µL subsample was used. This meant that for any given sample the sensitivity is a comparison of $1/70^{th}$ of a sample to $1/6^{th}$. This ratio could potentially be overcome through combining multiple DNA extractions; however, this is both lengthy, labour intensive, and carries the potential for reduction in DNA yield potential during the combination of DNA pellets.

Second, the accumulation of inhibitory agents such as humic acid, which is known to be in sediments and soils and can disrupt downstream applications as it behaves like nucleic acid during extraction (194). Potential additional steps could be added to the procedure in an attempt to remove additional inhibitors. For instance, a study in *crenarchaeota* used a two-phase agarose, one containing PVP (polyvinylpyrrolidone) in pulse field electrophoresis

to remove contaminating molecules, which resulted in pure high molecular weight DNA (195). The SDS preservation method is beneficial as it removes the need to process the samples immediately; however, if immediate sample processing is possible the Centrifugation method is preferred. The centrifugation method separates the cells from the sediment by density centrifugation (196). This allows for collecting the amoeba cells without accumulating the humic acid or other inhibitory particles that may be present within the sample.

SEDIMENT CHARACTERISTICS

In general, an inverse relationship is thought to exist between the particle size and nutritional quality of the sediments. Nutritional quality in this case being defined as the organic matter concentration and thus bacterial abundance within a sediment sample (197, 198). Larger particle sediments; i.e., gravel should therefore have a lower nutritional quality than smaller particle sediments i.e. mud (199). A study done on estuary benthic sediments associated with salmon seacages along the eastern coast of the United States, identified a threefold increase in microbial biomass associated with intensive feeding that lasted the duration of the production season (23). The study outlined the factors that potentially influence microbial population structure and biomass as: season, grain size and shape, carbon content, fluid flux over the benthic surface, disturbance of sediments, and animal-microbial interactions (23). Since it is known that *Neoparamoeba* and *Paramoeba* consume marine bacteria within marine sediments, it is likely that those factors also affect the abundance and distribution of amoebae in sediments.

It is a common practice to rotate seacages and fallow sites for periods of time after heavy production seasons (200-208). The process is meant to allow the seabed beneath cage sites to recover both in reduction of organic enrichment and in the repopulation of micro and macro fauna (200). There have been numerous studies considering the effects of fallowing on the restoration of benthic environments (201-209). The speed at which a site returns to a pre-farm state appears to be very site specific (210). The majority of the sediments received from British Columbia would be considered soft with most of the particles being in the mud/sand range (Table 12). There is evidence that this type of benthic site is more susceptible to organic enrichment leading to colonization by more opportunistic and resistant species (208). However soft sediment environments have been reported to return to a normal state post farming at a rapid rate (208). At farm sites along the north-eastern coast of Vancouver Island restoration occurred within a six-month period (201). In this study, *N. perurans* DNA was still detectable at a site that had been fallowed for sixteen months (Mahatta East) but not at a site that had been fallowed for twenty-four months (Cleagh Creek). It is not known whether this is due to the rate of benthic restoration making the habitat unfavourable for *N. perurans* after 24 months or due to *N. perurans* not being present at the Cleagh Creek site initially. Further work into understanding the factors dictating the distribution of *N. perurans* within sediment is needed to understand *N. perurans* risk in relation to fallowing farm sites.

Though the DNA preservation method sacrifices sensitivity for the ability to preserve and store samples, it remains useful in broad surveys where knowledge of the presence or absence of an *N. perurans* is desired, but processing availability is limited. It is also useful in cases where the sites may be too remote to allow for quick processing of sediments. The

differential centrifugation method has a high sensitivity, which lends itself well to the quantification of specific amoeba concentration for a more in-depth analysis of sites of interest. In addition, these results indicate that sediments may potentially be a reservoir for *N. perurans*.

Conclusion

This is the first study to conclusively identify sediment as a reservoir for *N. perurans*. The study identified *N. perurans* amoebae in sediments from both Canada and Australia in quantities 10-fold of those found in the water column in and around salmon cages. Identification of a reservoir has significant implications for the aquaculture industry, understanding where the amoebae are localized informs mitigation strategies. The amoebic DNA was found in both fallowed and active farm sites. This would indicate that amoebae have the ability to over-winter in the sediments and that fallowing a site may not be sufficient to ensure that re-infection will not occur.

In addition, this study introduced two methods for isolating and quantifying amoeba cells from sediment samples. These methods address both frozen samples or those that need to be preserved and fresh samples. These methods are versatile with application outside of *N. perurans* and as the DNA extraction protocol is universal it can be used on a variety of eukaryotic organisms including those associated with human disease.

CHAPTER 5 GENERAL DISCUSSION

5.1 THESIS OUTCOMES

This project set out to use molecular methods to investigate the relationships between geographically diverse samples of *N. perurans* and to explore possible environmental presence in the sediment. There are several conclusions that can be drawn from this study:

1. There are low levels of sequence heterogeneity when conserved gene sequences were compared using Multilocus sequence typing.
2. There are much higher levels of heterogeneity between samples when the whole genome profile is visualized using Random Amplified Polymorphic DNA
3. The difference between samples indicate that while *N. perurans* is a ubiquitous organism, there may be genetic changes occurring in "localized" populations
4. *N. perurans* DNA can be isolated from sediments associated with salmon farming and from sites that have been fallowed indicating that they are free living in this environment.
5. The analysis of samples indicate that amoebae occur in the sediment in higher concentrations than have previously and concurrently been reported in the water column.

This study has increased our knowledge of the relationship between *N. perurans* from geographically diverse outbreaks of Amoebic Gill Disease. It has led to a better understanding of potential transmission with the understanding that outbreaks are due to environmental interactions and not site to site transmission. In addition, the study identified sediment as a likely point of these environmental interactions due to the higher abundance

of amoebae present and has introduced new methods for the detection of *N. perurans* DNA in sediment. The following sections will explore these conclusions further, drawing comparisons with other disciplines and discussing various aspects of the chapters including limitations and future directions.

5.2 LIMITATIONS OF MLST AND RAPD

The MLST and RAPD studies were limited in several ways, firstly by the number of samples per country that could be obtained for the comparison. This initial analysis indicates that MLST could provide a universal typing scheme for *N. perurans* however before it is suitable for mass comparisons further work is required. One major limiting factor in gene selection in the MLST study was that primers could not be designed to be specific for just *N. perurans.* In part this was because many of the target genes, suggested in other MLST papers, only had bacterial representative sequences on GenBank which were determined to be unsuitable as the samples used in the analysis were not axenic(101, 109). With more genetic information becoming available, it is likely that more suitable and informative genes could be selected and used in MLST analysis. This has been the case in parasitic microbial genera, especially those that affect human health, that have undergone more extensive genomic exploration such as *Acanthamoeba*, *Trypanosoma, Trichomonas* and *Leishmania* (109, 110, 112, 128, 134, 135, 140, 171, 172, 211-215). From this previous genomic exploration several MLST schemes have been created and reformed as new information becomes available as in the case of *Trichomonas vaginalis* (214).

The addition of more *N. perurans* isolates from AGD outbreaks and subsequent clonal sequences from a) the countries compared in this study b) other AGD affected

countries c) emergent outbreaks and d) archival outbreaks would be particularly useful in resolving relationships between virulent *N. perurans* and inform both MLST and RAPD analysis. Both mixed and clonal samples should be considered. Clonal isolates are useful for resolving sequences where intragenomic variation occurs as is the case of length heterogeneity in the ITS of *N. invadens* (62), whereas mixed samples may show diversity in sequences that would not show up in a clonal culture.

In addition to collecting and testing new samples from current outbreaks, using archival DNA from both water (68, 119, 123) and outbreaks could help define the evolution and transmission patterns of *N. perurans.* Molecular methods have been used to characterize archival DNA in other pathogenic species (216). For example, archival sequences informed historical transmission incidents and helped to better understand the pattern of outbreaks of Yersiniosis in fin fish (102) and in the eukaryotic pathogen *Leishmania* (134). In another instance MLST was used to more rigorously address human-pet commonality in *E. coli* (ExPEC) clones and the study found that within a specific serogroup the genomic and virulence genotypes supported the potential of zoonosis between dogs and humans (216).

The study presented in this thesis demonstrated that there are genetic differences between *N. perurans* samples. At present RAPD is a more informative tool as a potential typing method based on the amount of variation between samples detected using this method. MLST, however still has potential as a typing tool for *N. perurans* but will require more work to create a reliable gene set. Future studies should continue to build upon the analysis presented in this study by refining both the RAPD analysis and the MLST analysis.

5.3 POTENTIAL CAUSES OF *N. perurans* ISOLATE VARIATION

One of the main considerations of this thesis was to compare samples of *N. perurans* from geographically diverse AGD outbreaks. The initial study using MLST showed that the samples were highly conserved across the six tested housekeeping genes but could be differentiated based on geographic origin from one or two polymorphisms. When combined with the results from the RAPD analysis it became apparent that these samples were not genetically identical. There were evident larger scale intergenomic differences between samples. The lack of predicted founder in the eBURST analysis and the geographical pattern in the MLST and RAPD analysis would suggest that there are globally "local" populations within the ubiquitous *N. perurans,* which due to unknown environmental influences have opportunistically turned to parasitism.

One major limitation for both the MLST and RAPD studies was the lack of axenic cultures. Axenic cultures allow for a more defined and controlled set of conditions that contribute to easy reproducibility (217). This has become even more important as research moves towards routine molecular work and 'omic' technologies (217). Axenic cultures have been achieved in a range of amoeba genera including *Acanthamoeba, Entamoeba* and *Naegleria* as well as protozoan fish parasites such as *Philasterides* and *Spironucleus* (218-222). Axenic cultures allow for the control of outside contaminations from bacteria in proteomic studies, mRNA and metabolite profiles along with enhanced ability to characterize cell physiology, all of which are important factors in understanding amoebic genomic and proteomic profiles for vaccine creation (217, 223). Attempts were made to axenise culture of *N. perurans* at the beginning of this study, however they were

unsuccessful (Appendix 1). It was possible to visually eliminate bacteria from the cultures using high doses of Penicillin-Streptomycin-Neomycin (Figure A.1.3, Appendix 1). The culture however showed reduced growth and, in many cases, declines in amoebae numbers (Appendix 1). The inability to create axenic cultures and the diverse types of preparations of the other geographical samples meant that the quality of DNA extracted from each preparation was different. This may have affected the quality of sequencing and thus lead to shorter MLST segments. In addition, though checks were run to ensure that bacterial bands were not counted in the RAPD results, it is impossible to ensure that co-migration of segments did not occur which would impact the ability to excise and sequence specific bands of interest.

5.4 RAPD VARIATION AND VIRULENCE

One area of particular interest for further research is the relationship between the parasome and amoebae nucleus as it is not known how much the genetic material of the parasome influenced the RAPD results. There is evidence that the presence of endosymbionts can cause population variation in RAPD analysis (224). A study on population heterogeneity in the endoparasitoid of silkworms, the uzifly (*Exorista sorbillans*), in south India found that the population was distinguishable by the type of *Wolbachia* endosymbiont the uzifly carried (224). *Wolbachia* can be differentiated into supergroup A, B or a combination of A and B which was used to determine geographic origin (224). *Neoparamoeba'*s endosymbiont was incorporated into the genus from a single evolutionary endosymbiosis event and transferred vertically from mother to daughter in an obligate relationship (65). As the parasome is obligate it would not be as discriminatory in

influencing the RAPD analysis as *Wolbachia* is in uziflies. The Kinetoplastida group from which the parasome originated, contains many disease-causing members including known fish parasites such as *Trypanoplasma borreli* an extracellular blood parasite of cyprinid fish (225) and *Ichthyobodo necato*r, an ectoparasite of a wide range of fish species including salmon (226). The role in which the parasome may influence virulence and contribute to the genetic diversity observed is not yet known and therefore its influence cannot be discounted (66). In *N. pemaquidensis,* the parasome and host share a close metabolic association indicating the communication between the parasome and host still actively occurs (66). Given the parasome's evolutionary link to known parasitic species, there is potential that the parasome contributes genes expressed in virulence which may influence the differences in the avirulent versus virulent RAPD patterns.

N. perurans has been shown to lose virulence after three years in culture (137). The clonal isolate originated from the same source as the wild Tasmania isolates, farm infected fish (137). Once isolated in 2011, it was kept in culture for four years and tested periodically in fish trials to assess its pathogenic quality prior to being shown avirulent in 2015 (137). The isolates were then maintained in culture before being used in the studies presented in this thesis (137). It is interesting to note that there are observable differences in the all the RAPD pattern between the clonal and wild Tasmanian *N. perurans* isolates. One potential cause of these differences is virulent status, as this has been demonstrated in other pathogenic amoebae (217). Known symptomatic and asymptomatic strains of E. histolytica displayed different RAPD patterns which were linked to their virulence status (217). It would be beneficial to excise and sequence some of the bands present in the virulent strains and not in the clonal strains and determine if they correspond with genes known to be associated

with disease. Cysteine proteases may be ideal targets to begin with as they have been linked to virulence in *E. hystolytica,* contributing to intestinal invasion (222).

5.5 GEOGRAPHICAL ISOLATION AS A POTENTIAL CAUSE OF *N. PERURANS* ISOLATE VARIATION

One of the most probable causes of the variation observed in the RAPD analysis is geographic isolation. In a microsatellite study on the global patterns of gene flow in Pseudo-nitzschia pungens, geographical clustering patterns were observed (227). The authors suggested that this type of clustering pattern indicated that gene flow and migration rates were not strong enough to determine the sampling locations as one panmictic population (227). So though determined to be the same species, their data suggested that long-distance dispersal potentially occurred, but it was not frequent enough to counteract the effects of population differentiation (227). Even though *N. perurans* is not known to sexually reproduce, it is possible that a similar scenario occurs where dispersal/ migration is not strong enough to maintain a 'global' population across locations that are the most geographically distant. Though geographical isolation is likely, the analyses cannot determine that transfer between populations is not occurring. In a study on population genetic differentiation in giant Kelp (*Macrocystis pyrifera*), both the spatial distribution and habitat continuity played major roles in modelling predicted levels of differentiation (228). Similar to the phenomenon observed in giant Kelp along the coast of California, USA where proximity and habitat continuity lead to greater genetic diversity, greater variation was observed amongst *N. perurans* samples that were geographically close to each other. This can been seen in the RAPD grouping patterns of the European *N. perurans* samples (228).

This could possibly be explained by the relative closeness of the isolated non-clonal 'populations' compared. One possibility is that storm events and/or currents have the potential to create mixing of these "local" populations of *N. perurans* on a more global scale which has been seen in N. invadens (153). Outbreaks of Sea urchin paramoebiasis caused by N. invadens along the eastern coast of Canada have been linked to tropical storm events and hurricanes occurring in the North Atlantic and rises in sea temperature (153). These results however need to be interpreted with caution as the number of samples from each geographic location was limited and thus it is dangerous to draw too much of a conclusion about their apparent relationships.

5.6 MLST, RAPD AND THE ENVIRONMENTAL SAMPLES

In addition to including more isolations from AGD outbreak and new genes into the MLST analysis, applying MLST and RAPD analyses to samples from water near and within seacages along with sediment and biofouling could be particularly interesting. A study comparing environmental and clinical isolates of *Legionella pneumophila* using a similar typing method to MLST, RFLP, found that the clinical isolates showed less type diversity than the environmental isolates (229). The authors concluded that the clinical isolates were comprised of a subset of the environmental types indicating that not all types of *L. pneumophila* found in the environment caused disease (229).

N. perurans DNA has now been isolated from a variety of environmental and invertebrate sources including the water column, sediment and bio-fouling organisms (Chapter 4, 68, 118, 119, 123). It is not known yet however, whether the amoeba isolated from sediment have the ability to cause AGD outbreaks in Atlantic salmon. For instance, in

the case study from Canada presented here, amoebae were isolated from sediments associated with seacages in which salmon were negative for AGD (Chapter 4). Sediment was also positive in fallowed environments that had not been actively farmed for at least 70 weeks. There are several known instances, especially for bacteria, where not all strains of the same species cause disease (229-232). There is evidence that both pathogenic and non-pathogenic strains of the same species also occur in *Entamoeba histolytica* in the environment (231). Though avirulance has been demonstrated in culture, further environmental surveys and subsequent laboratory challenges should be undertaken to identify if this is the case for *N. perurans* isolated from the sediments and water column associated with salmon that have not developed AGD.

5.7 BENTHIC SEDIMENT AS A POTENTIAL HABITAT FOR *N. PERURANS*

When considering the abundance and dispersal ability of protists, a generalization has been made that any given mL of water would contain 10^3 cells and that this number could be magnified 100 fold in sediment (233). Higher numbers of small organisms in any substrate are needed to ensure that dispersal occurs on unlikely/ unpredictable events (233). This would appear to hold true for *N. perurans.* Though the Australian case study (Chapter 4) was limited in size and sampling distribution, water and sediment samples were both positive at one location allowing for a more direct comparison. In 1 L of water less than a single amoeba was detected using qPCR whereas in 20 g of sediment 25 amoebae cells were detected using qPCR. When scaled up to the equivalent of one litre there are an estimated 1,250 amoebae cells per kilogram. These numbers are far greater than those

estimated in any quantitative study of water samples around Tasmania (68, 119, 123). The highest reported number of amoebae per litre of water was 62.3 cells, from a heavily infected site (AGD prevalence 64%) (119). Even if the most conserved amoebae numbers returned from this one sampling in the case study (Chapter 4) are considered, there would still be an estimated 100 cells per kilogram which is more than the highest reported in the water column.

Amoebae are an important component of the protozoan contingent in benthic food webs. Naked Gymnamoebae, a subclass of lobose amoebae to which *Neoparamoeba* belong, have been found in high abundance in surface sediments (234-236). Amoebae, such as *Vannella* sp., tend to exhibit the most diversity in the top 1 cm of sediment and have been found in oxygenated and anoxic sediments (237). This includes sediment that change between oxygenated and anoxic due to mats of sulphur bacteria (237). These bacteria are known to be associated with aquaculture sites for their role in the degradation of organic materials making up the waste biproducts from production (238). Each type of aquaculture produces different types of waste in different proportions, but generally include, food and faecal matter, metabolic/ pesticide/antibiotic/fertilizer residues, and organic material from moulting and collapsing algal blooms (238, 239). Providing ample material for large active bacterial populations which may act as a readily available food source for amoebae. One study conducted on the grazing potential of a variety of amoebae from the U.K. found that the bacterial consumption rates indicate that amoebae are important grazers within this habitat (240).

The study from the U.K. exhibited patchy distribution with samples ranging from absent to 66 amoebae per cm-3 (240). The same type of patchy distribution was observed in the

sediment surveys in Tasmania of *Neoparamoeba* (82, 83) and for *N. perurans* (Chapter 4), particularly sediment samples from Tasmania where amoeba number could be calculated.

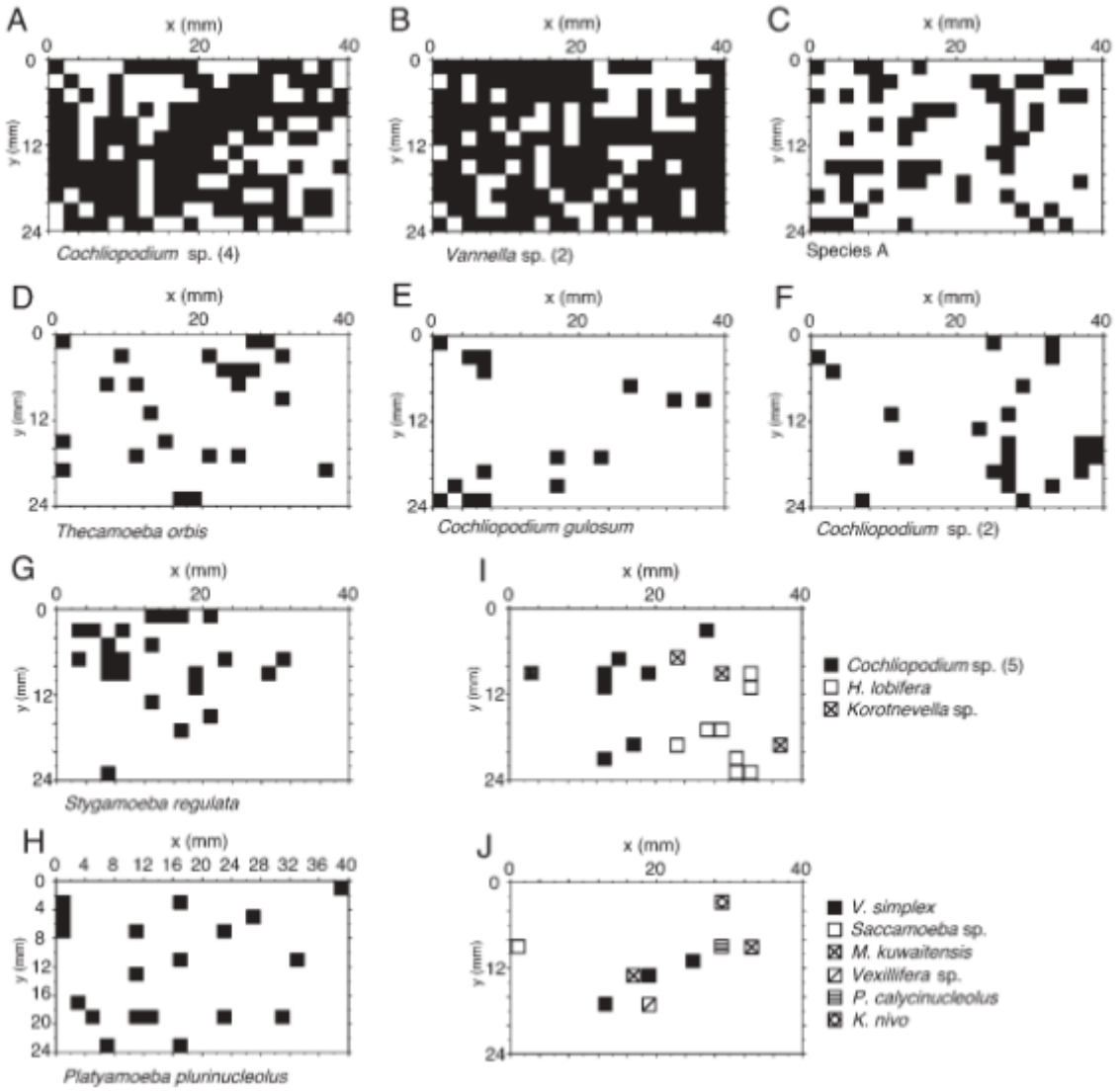

Figure 5.1 Representation of distribution patterns of amoeba species from a sub sample taken from bottom sediments in Denmark. Each square grid represents 2 x 2mm. (figure from Smirnov and Thar 2003).

The type of distribution patterns was studied by taking a benthic sediment sample from Denmark and dividing the sample into 2x2x2 mm^3 subsamples to explore abundance and

distribution of amoeba species (241). The study found that the amoebae displayed heterogeneous distribution that may be linked with microhabitats (Figure 5.1) (241). Different species exhibited different distribution patterns falling into three categories: random, aggregated and equally spaced with only one species being equally spaced (241). The study determined that due to the heterogeneity it was likely that under sampling would lead to misrepresentation of species and their abundance and stressed the importance of lasting observation and extensive sampling in reliable amoeba biodiversity studies (241).

DNA of several species of *Neoparamoeba* and *Paramoeba* (*P. atlantica, N. longipodia, N. pemaquidensis and P. shaudinni*) have been identified from benthic sediments after culturing (63, 74, 83, 89). It is possible the *N. perurans* could form complexes of sibling species leading to instances of co-infections. Sibling species complexes have been reported in other fish parasite such as *Ichthyobodo necator* (242). Sibling species complexes are aggregations of morphologically indistinguishable and molecularly distinct species (233, 243). As in the study on amoebae distribution, there is evidence that species share geographical and environmental niches on a larger scale as well as co-infect hosts (241, 243-245). This may partially explain why *N. pemaquidensis, N. branchiphila* and *N. perurans* have all been isolated from gills of AGD infected fish when *N. pemaquidensis* and *N. branchiphila* exhibit no pathogenic effect (16, 22, 59).

Further studies should survey sediments not only for the presence and abundance of *N. perurans* but also for other species, namely *N. pemaquidensis* and *N. branchiphila* which were not targeted in this thesis as specificity of *N. perurans* was desired. Broader and cross season surveys should be undertaken to better understand the abundance and distribution of *N. perurans* in sediment and ascertain whether seasonality affects population density. It

would be especially interesting to survey sediments from under a variety of farm site conditions: fallowed, active, AGD positive and AGD negative sites. Additional laboratory infection trials should be undertaken using samples from sediment and bio-fouling organisms to confirm the infectivity potential of these types of samples. There is also potential to combine the DNA extracted from these sediment surveys with others including virulent samples in MLST/RAPD analysis. This would allow a better understanding of differences if any that may occur between environmental and virulent samples since not all seacages where *N. perurans* were in the sediment developed AGD. This in turn will better inform the associated environmental risks for salmon farms, helping them to create AGD mitigation strategies based on the determined risk of any one particular site.

5.8 IMPLICATIONS FOR PARASITE/ENVIRONMENT INTERACTIONS

Understanding parasite transmission and reservoir populations plays an important role in designing management strategies for potential risk associated with pathogens (246). For many marine amoebae, such as with *N. perurans*, population reservoirs are not well understood and transmission from wild fish to seacage fish are not well monitored which can impact the effectiveness of management strategies (46, 51, 247). Though recent studies have suggested that once infected in cage systems, Atlantic salmon are the most important reservoir, this does not explain initial infection (118, 248). It is possible that sediment is a primary habitat for *N. perurans* and that additional amoebae species may populate the sediments around salmon seacages and biofouling. It is interesting to note that during culturing experiments (Appendix 1, Figure A1.5) on several occasions *N. perurans* cells formed aggregates of hundreds of cells. In one instance the aggregation was dense enough

to be seen without a microscope (Appendix 1, Figure A1.5 B). This ability to form multicell aggregates may help explain the vertical movement of amoebae from sediment to seacages and depth differences observed within seacages (119, 123). It is not yet known how amoebae move within the water column.

More research should be undertaken to better understand if and how amoebae settled in sediment have the potential to infect fish. In general, other parasites found in sediment are transferred in three ways. 1) Through sediment dwelling intermediate hosts, 2) through water currents and 3) through sediment disruption (6, 173, 249). *N. perurans* does not have an intermediate host and has been shown to cause disease in laboratory challenges using water containing the amoebae which makes the first form of transmission impossible (93). A recent study however, found that *N. perurans* can infect known bottom dwelling fish species, namely lumpsuckers, and the infection is asymptomatic (248). Lumpsuckers, and other cleaner fish are often found naturally associated with seacages or are introduced into salmon seacages to help to control other parasites such as sea lice (17, 250). Therefore, one possible scenario for infection is that wild fish disrupt sediments and become infected with *N. perurans* before coming in contact with seacages. This hypothesis, however, requires further study and may be quite difficult to accurately represent in a laboratory challenge system with our present knowledge of *N. perurans* genomics and typing methods. However, it may be possible using the differential centrifugation method outlined in chapter four (prior to the lysis step) to collect viable amoebae from sediments to spike a tank of lumpsuckers prior to transfer into a tank with naïve salmon.

Another strategy that has been developed to reduce parasite transmission is the movement of seacages and changes in site selection criteria (249). In particular, the distance

of the cage from the shore and the associated depth have been shown to be highly effective in minimizing parasite infections (173, 176, 249). In Tilapia moving the seacages away from the shore into deeper water in the ponds decreases the chances of helminth transmission (173). Similarly in Southern Bluefin Tuna offshore farming/ ranching compared to inshore sites lead to increased fish health and decreased parasite load (176). Though *N. perurans* is a facultative parasite, the same strategies may be effective. The move to offshore seacages has already begun to occur in certain countries, such as Norway, where the availability of coastal sites is limited (250, 251). It is not yet known how this affects the severity of AGD. There have not yet been any comparative studies done on the rate and intensity of AGD infections between offshore and inshore farms which would be of interest.

5.9 ADVANCES IN ENVIRONMENTAL DETECTION OF *N. PERURANS*

The second key issue outlined in this thesis, methods to detect *N. perurans* DNA in sediments was addressed in chapter 4. Both methodologies, DNA preservation and differential centrifugation, identified the presence of *N. perurans* DNA in benthic sediments associated with farms. One limitation of the sediment study was that the sampling and processing methods were different for Canada and Tasmania. The equipment for the samplings was dictated by the farm companies where the samples were taken, and the same equipment was not used at both locations. It was therefore not possible to compare the sampling methods and investigate how differences in sampling methods may impact detected abundance. It would be useful to compare sampling methods in the same artificially controlled environment where sediment is spiked with a known number of non-biological agents. This would allow for the accurate comparison of the number of amoebae

picked up by each device and to compare the volume of displaced sediment by each method.

In addition to the proven usefulness for *N. perurans*, both DNA extraction and analysis methods may have potential applications for the detection of other soil/sediment borne pathogens. There are several pathogenic protozoan species that can be found in soil that have human health impacts such as, *Cryptosporidium parvum, Giardia intestinalis* and *Entamoeba histolytica* (252). The DNA preservation method could be applied to samples that needed to be preserved prior to analysis in the laboratory. In many cases parasitic diseases are present in developing countries where quick access to laboratories can be limited (253). For example, the prevalence of *G. intestinalis* ranges from 20% - 40% in developing countries where locations are potentially too far from a laboratory. Though prevalent in developing countries, *G. intestinalis* is still present in developed countries at a prevalence of 2% -5% (253-256). Since there is still a presence of *G. intestinalis* in developed countries, the differential centrifugation method may prove useful as quick processing is likely and high sensitivity is optimal given the likelihood of lower concentrations (253, 256). This research has created a platform for future studies that can use large volumes for a variety of industries for sediment/soil surveyance in association with known infectious parasitic diseases.

5.10 SIGNIFICANT CONTRIBUTIONS

The MLST and RAPD studies are the first of their kind directly comparing *N. perurans* DNA from infected Atlantic salmon gills sourced from geographically diverse countries. These studies have provided preliminary evidence that the trend of increasing outbreaks is

due to changes in environmental factors, not spread of the pathogen. This forms the basis from which future comparative studies can be built upon through the addition of new samples of *N. perurans* from infected gills, new environmental isolates and genome sequencing. Combined with whole genomic studies on sister species and with the growing field of bioinformatics it is possible to explore further the differences observed between these geographic samples particularly where virulence is concerned. This will potentially lead to the development of better treatment and vaccine targets for AGD.

The thesis also introduces the first conclusive evidence that *N. perurans* is present in sediments both in stocked and in fallowed farming sites. It also provides evidence that *N. perurans* amoebae congregate in detectable numbers in both North American and Australian sediments. It is therefore likely that local populations of *N. perurans* are present in sediments globally and have yet to be detected. This knowledge presented in this thesis has major implications for aquaculture management of disease. For instance, rotation of the cages within the same farm site may not be enough to minimize the risk of re-infection due to the widespread presence of *N. perurans* in sediments.

Beyond the implications for industry and management of farm sites, the sediment studies have implications for research. This could be particularly important for aquaculture trials that are using water from benthic sources. Water drawn from near the bottom would have a higher chance of including pathogenic amoeba which if used untreated, may confound trials by introducing un-planned pathogens or additional pathogen numbers.

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APPENDIX 1 SOURCING ADDITIONAL NUTRITION SOURCES FOR REDUCED BACTERIA AND ENHANCED GROWTH IN *NEOPARAMOEBA PERURANS*

A1.1 INTRODUCTION

The first isolation and description of the amoebae from the gills of AGD-affected fish occurred in 1988 and marked the characteristics as belonging to the genus *Paramoeba,* which was known to contain parasitic members (1). The authors identified the species as *P. pemaquidensis* based on the size of the nucleus and parasome and other untrastructural characteristics. In this initial isolation, liqiud cultures were used and enriched with fetal calf and chicken serum whilst being treated with antimicrobial agents (neomycin, kanamycin, novobiocin, penicillin, streptomycin and nystatin)(1). Agar cultures were then established from these liquid cultures using a malt yeast agar with an overlay of *Klebsiella* bacteria. In part due to the previously understood mixed aetiology of AGD, amoebae associated with AGD lesions on the glls have been continuously reisolated and cultured since the initial outbreak for research purposes. Marine amoebae are typically cultured following a basic isolation technique (2-5) which involves inoculation of small amounts of water, sediment or tissue onto an agar surface covered with a bacterial overlay to serve as a food source (6). Over time the culturing procedure for marine amoebae was adapted and a bacterial overlay was no longer added as it was found that even host-derived amoebae bring bacteria with them during isolation (6-8). Prior to 2012 the only AGD-associated *Neoparamoeba* spp. that could be cultured in such a manner were *N. pemaquidensis* and *N. branchiphila* (9), neither of which could induce AGD in tank-reared Atlantic salmon (See 10). The need to have access to large numbers of infective amoebae led to the development of a modified protocol and

N. perurans is now routinely cultured following the method outlined in Crosbie et al. 2012 (11). Amoeba are isolated from gills using the technique of Morrison et al. (2004) then partially purified cells are inoculated onto malt yeast agar plates, overlayed with filtered seawater and incubated at 18°C in Australia (11). The advent of *N. perurans* cultures secured the supply of large numbers of cells at any time for further research (11). However, there are still issues with the culturing of *N. perurans*, which presents potential problems for downstream analysis. Since *N. perurans* has the ability to bring bacteria from the environment, it can be difficult to control their growth or even exclude them. Although bacterial growth can be controlled by constant subculturing, this is labour intensive and axenic cultures would be preferred (6). The ability to axenically culture *N. perurans* would assist with further research, including molecular analysis. At present initial DNA extraction from cultured amoebae is inefficient and cells are lost during removal from the agar-based culture medium and during the washing processes that remove the excess bacteria in culture thus limiting the yield and purity of the DNA. This has a cumulative effect when considering that this DNA is needed either for quantity sensitive methods such as qPCR or for typing methods (such as Multi locus sequence typing and Random Amplified Polymorphic DNA) that require clean, non-degraded DNA. To alleviate the possibility of ambiguous results in some molecular techniques such as sequencing RNA, it is important to produce axenic cultures (12), it also preferable to culture amoebae in liquid medium to avoid issues of agar interference is DNA extractions and access to amoebae.

Axenic cultivation is not straightforward but can be achieved through a variety of methods (13, 14) The two most successful methods, continuous liquid culturing and continuous agar subculturing, have been successful in other parasitic amoebae species

including *Acanthamoeba* and *Entamoeba* and as well in a variety of other marine protozoan species (15-18). In the past, other amoebae species have shown the ability to be cultured both on agar plates and in liquid culture (6, 19, 20). *Naegleria* sp. is one such species where liquid cultures have been useful for optimal growth conditions in a sometime difficult species to culture (6, 19, 20). Antibiotics can be used, and penicillin and streptomycin have been successfully applied to growth media for other amoeba species including *Acanthamoeba spp.* and *Naegleria* spp.

The aim of this study is to find alternative sources of nutrition allowing for short and long-term axenic cultures of *N. perurans* using various media and commercial antibiotic mixtures to allow for easier and non-ambiguous study of the amoeba at the molecular level.

A1.2 MATERIALS AND METHODS

A1.2.1 AMOEBA COLLECTION

Amoebae were isolated from the gills of AGD-affected Atlantic salmon housed in tanks at the University of Tasmania, Australia, following the protocol from Morrison et al., (2004) with minor adjustments (21). Briefly, the entire gill was excised and dissected into individual arches in seawater. Amoebae were removed from the gill by agitating the gill arches in 25 mL distilled water in 50 mL tubes for 30 s then adding of 25 mL 70ppt filtered saltwater to normalize the salinity to 35ppt. The liquid was then decanted into petri dishes and left for 1h to allow the amoebae to attach after which the plates were washed 4-5 times vigorously with 0.2µm filtered seawater to remove unattached debris. The amoebae were detached by adding 15 mL of distilled water for 5-10 s before the addition of an equal

volume of 70ppt (to bring the salinity to 35ppt) and detached amoebae were poured into 50 mL centrifuge tubes.

A1.2.2 MEDIA TRIALS

Four trials were carried out with different combinations of media, primarily liquid media consisting of: malt yeast seawater (MYSW, 0.01% malt, 0.01% yeast, seawater at 35ppt), Leibovitz culture medium (L-15) supplemented with the amino acid, L-glutamine (1.025 mL L-glutamine + and 48.975 mL L-15), or 0.2 µm filtered seawater. Colloidal silver was used in each medium as an anti-flagellate and anti-ciliate agent and was used at concentrations of approximately 12ppm. The antibiotic solution used was a commercial preparation of penicillin (5,000 units/mL), streptomycin (5 mg/mL), neomycin (10 mg/mL) (PSN) (Sigma-Aldrich, NSW). Amoebae used in each trial were single cell selected and counted into 60 µl filtered seawater in a 96 well plate until the initial start number (i.e. 20) was achieved to ensure the correct number per well prior to be transferred into the experimental container where they were counted again to ensure none were lost.

A1.2.3 TRIAL ONE: DETERMINATION OF BASE CULTURE TYPE AND TEMPERATURE FOR ENHANCED GROWTH

Two media types were compared with a seawater control. Each media formulation contained the following:

1) Leibovitz tissue culture (L-15) made by the addition of 0.02 % L-15 to a mixture of 50 % Colloidal silver seawater and 50 % 0.2µm filtered seawater. 1xPSN was added to control bacterial growth.
2) Malt yeast in seawater (MYSW) made by the addition of 0.02 % solid malt yeast dissolved in a mixture of 50 % Colloidal silver seawater and 50 % 0.2µm filtered seawater. 1xPSN was added to control bacterial growth.
3) Seawater control consisting of a mixture of 50 % Colloidal silver seawater and 50 % 0.2µm filtered seawater. 1xPSN was added to control bacterial growth.

Three 12 well plates were prepared for each formulation. Each well contained 2 mL of media and 20 amoebae per well. One plate was placed at each of the following three temperatures, 7°C, 10°C and 18°C to test the optimal temperature for growth. Daily counts were conducted over a period of five day using light microscopy at 10x magnification.

A1.2.4 TRIAL TWO: ADDITIVES TO L-15 MEDIA FOR ENHANCED GROWTH

Three additives were tested against a control. Based on trial one, L-15 media in seawater was the preferred base media and used for future trials. Each media formulation contained the following:

1) Foetal bovine serum (FBS) made by the addition of 250 µL of 5 % FBS to 3.5 mL of 50 % Colloidal silver seawater and 50 % 0.2µm filtered seawater plus 1 mL L-15 medium. 250 µL, equivalent to 5x, PSN was added to control bacterial growth.

2) Lysed amoebae extract made by the addition of 250 µL lysed amoebae to 3.5 mL of 50 % Colloidal silver seawater and 50 % 0.2 µm filtered seawater plus 1 mL L-15 medium. 250 µL, equivalent to 5x, PSN was added to control bacterial growth.
3) Gill/cutaneous mucus made by the addition of 250 µL gill or cutaneous mucus to 3.5 mL of 50 % Colloidal silver seawater and 50 % 0.2 µm filtered seawater plus 1 mL L-15 medium. 250 µL, equivalent to 5x, PSN was added to control bacterial growth.
4) Seawater control consisting of 4.750 mL of 50 % Colloidal silver seawater and 50 % 0.2 µm filtered seawater. 250 µL, equivalent to 5x, PSN was added to control bacterial growth.

One six well plate was prepared for each formulation. Each well contained 5 mL of media and 20 amoebae per well. Plates were placed at 10°C as determined as the optimal temperature for growth. Counts were conducted every three days over a period of six day using light microscopy at 10x magnification.

The lysed amoebae were prepared from approximately 100,000 cells. Cells were concentrated by centrifugation at 400xg for 5 min. The supernatant was drawn off and 5 mL of distilled water was added and the concentrated cells and left for 2 h to lyse. After the 2 h incubation, the tube contents were vortexed to break apart the cell remnants and passed through a 0.2µm syringe filter. The fish mucus was collected by placing a salmon which had succumbed to amoebic gill disease in 0.2µm filtered seawater for at least 1h, after which the mucus was scraped from the skin and gills with a L-shaped plastic stick, collected and passed through a 0.2um syringe filter.

A1.2.5 TRIAL THREE: FISH MUCUS AND FBS CONCENTRATIONS FOR ENHANCED GROWTH

Eight media formulations were compared. From trial two, FBS and Fish mucus were chosen for further experimentation. Each media formulation contained the following:

1) 10% FBS + Fish Mucus made with 0.01g g FBS dissolved in 100 µL L-15 medium plus 66 µL Colloidal silver seawater, 24 µL Fish mucus and 10 µL PSN (5x)
2) 5% FBS + Fish Mucus made with 0.005g FBS dissolved in 100 µL L-15 medium plus 66 µL Colloidal silver seawater, 24 µL Fish mucus and 10 µL PSN (5x)
3) 2% FBS + Fish Mucus made with 0.002g FBS dissolved in 100 µL L-15 medium plus 66 µL Colloidal silver seawater, 24 µL Fish mucus and 10 µL PSN (5x)
4) 0% FBS + Fish Mucus made with 100 µL L-15 medium plus 66 µL Colloidal silver seawater, 24 µL Fish mucus and 10 µL PSN (5x)
5) 10% FBS made with 0.01g g FBS dissolved in 100 µL L-15 medium plus 90 µL Colloidal silver seawater and 10 µL PSN (5x)
6) 5% FBS made with 0.005g FBS dissolved in 100 µL L-15 medium plus 90 µL Colloidal silver seawater and 10 µL PSN (5x)
7) 2% FBS made with .002g FBS dissolved in 100 µL L-15 medium plus 90 µL Colloidal silver seawater and 10 µL PSN (5x)
8) 0% FBS made with 100 µL L-15 medium plus 90 µL Colloidal silver seawater and 10 µL PSN (5x)

One twelve well plate was prepared for each formulation. Each well contained 200 µL of media and 20 amoebae per well. Plates were placed at 10°C as determined as the optimal temperature for growth. Counts were conducted every day over a period of five days using light microscopy at 10x magnification.

A1.2.6 TRIAL FOUR: ANTIBIOTIC ADDITIVES FOR REDUCTION OF BACTERIA TOWARDS AXENIC CULTURES

Ten concentrations of PSN (penicillin (5,000 units/mL), streptomycin (5 mg/mL), neomycin (10 mg/mL)) were tested against a control. FBS was chosen as the preferred additive and ten % as the preferred concentration. Each media formulation contained the following:

1) 1x PSN made with 100 µL L-15 medium and 0.01g FBS (10 %) dissolved in 98 µL plus 2 µL PSN.
2) 2x PSN made with 100 µL L-15 medium and 0.01g FBS (10 %) dissolved in 96 µL plus 4 µL PSN.
3) 3x PSN made with 100 µL L-15 medium and 0.01g FBS (10 %) dissolved in 94 µL plus 6 µL PSN.
4) 4x PSN made with 100 µL L-15 medium and 0.01g FBS (10 %) dissolved in 92 µL plus 8 µL PSN.
5) 5x PSN made with 100 µL L-15 medium and 0.01g FBS (10 %) dissolved in 90 µL plus 10 µL PSN.
6) 6x PSN made with 100 µL L-15 medium and 0.01g FBS (10 %) dissolved in 88 µL plus 12 µL PSN.
7) 7x PSN made with 100 µL L-15 medium and 0.01g FBS (10 %) dissolved in 86 µL plus 14 µL PSN.
8) 8x PSN made with 100 µL L-15 medium and 0.01g FBS (10 %) dissolved in 84 µL plus 16 µL PSN.

9) 9x PSN made with 100 µL L-15 medium and 0.01g FBS (10 %) dissolved in 82 µL plus 18 µL PSN.
10) 10x PSN made with 100 µL L-15 medium and 0.01g FBS (10 %) dissolved in 80 µL plus 20 µL PSN.

One twelve well plate was prepared for each formulation. Each well contained 200 µL of media and 20 amoebae per well. Plates were placed at 10°C as determined as the optimal temperature for growth. Counts were conducted every two days over a period of five days using light microscopy at 10x magnification. The amoebae counts were followed by a refresh of the media done by removing 100 µL and replacing it with 100 µL of a new formula preparation. 10 µL of the removed 100 µL was placed in 1 mL Lysogeny broth (LB) incubated at room temperature (Approximately 24°C and at 37°C). Cultures were said to be nominally axenic when there were no visibly moving bacteria present under light microscopy at 40x magnification coupled with no bacterial growth in a saltwater LB media after incubation for a minimum of 2 weeks.

A1.2.7 SUSTAINABLE LIQUID CULTURES

To establish sustainable liquid cultures, a trial was carried out using formalin-inactivated *Escherichia coli* bacteria as an additional food source as growth using the above additives was minimal. The bacteria were prepared as follows: briefly, bacteria were grown overnight in 50 mL LB. The bacteria were pelleted and resuspended in a 0.01% formalin solution for 30 min. The cells were pelleted and resuspended 4 times in PBS to remove residual formalin and then resuspended in 0.2 µm filtered seawater. Sterility of the

preparation was verified on sheep blood agar plates incubated at room temperature for 2 weeks.

The cultures were prepared as follows:

1) 10 % FBS dissolved in 10 mL 35 ppt salt adjusted L-15 medium combined with 8 mL Colloidal silver sea water and 4 mL (10x) PSN and 200µl formalin killed bacteria (approximately 360,000 cells).

Six petri dishes were prepared. Each dish contained 40 amoebae and were incubated at 10°C. After the initial 10x PSN dose, a reduced (5x) PSN dose was added every 2nd day for the first week, then weekly with the media changes. Amoebae number, size and morphology were monitored daily using 10x and 40x light microscopy.

A1.2.8 STATISTICAL ANALYSIS

For each trial one way ANOVAs were carried out combined with Tukeys multiple comparison test to determine significant differences. ANOVAs were calculated using the GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

A1.3 RESULTS

A1.3.1 TRIAL ONE: DETERMINATION OF BASE CULTURE TYPE AND TEMPERATURE FOR ENHANCED GROWTH

In all temperatures numbers of *N. perurans* were significantly higher in L-15 media ($P<0.0001$) than in MYSW or the colloidal silver seawater control (**Error! Reference source not found.**). The amoebae numbers in L-15 and MYSW were the same for the first two days of the trial. Amoebae numbers in L-15 however, increased and stayed significantly greater than the other two treatments for the remainder of the trial. In addition, although L-15 and MYSW were both capable of maintaining low levels of growth throughout the trial, in MYSW incidences of fungal contamination were higher and spread more rapidly. Therefore L-15 was chosen as the preferred media.

When all three temperatures were compared, amoebae numbers were consistently lower in the 7°C treatment when compared to 10°C and 18°C. Though the amoebae did not replicate as fast at 7°C, there was also no increase in bacterial contamination observed which was seen in both 10°C and 18°C. Amoebae numbers were highest in 18°C, with slightly lower numbers occurring at 10°C. Replicates at both 10°C and 18°C were lost due to bacterial contamination. On day 4 at 18°C MYSW was down to 9 replicates. By day 5 MYSW had only 5 replicates remaining and L-15 lost 3 replicates. 10°C had a higher replicate survival when compared to 18°C with MYSW having 8 replicates clean enough to count at the end of the trial on day 5. Overall, cultures at 18°C were observed to have quicker rates of bacterial growth when compared to 10°C and 7°C. Therefore 10°C chosen as the optimal culture temperature as it controlled contamination growth while still allowing for relatively higher numbers of amoebae.

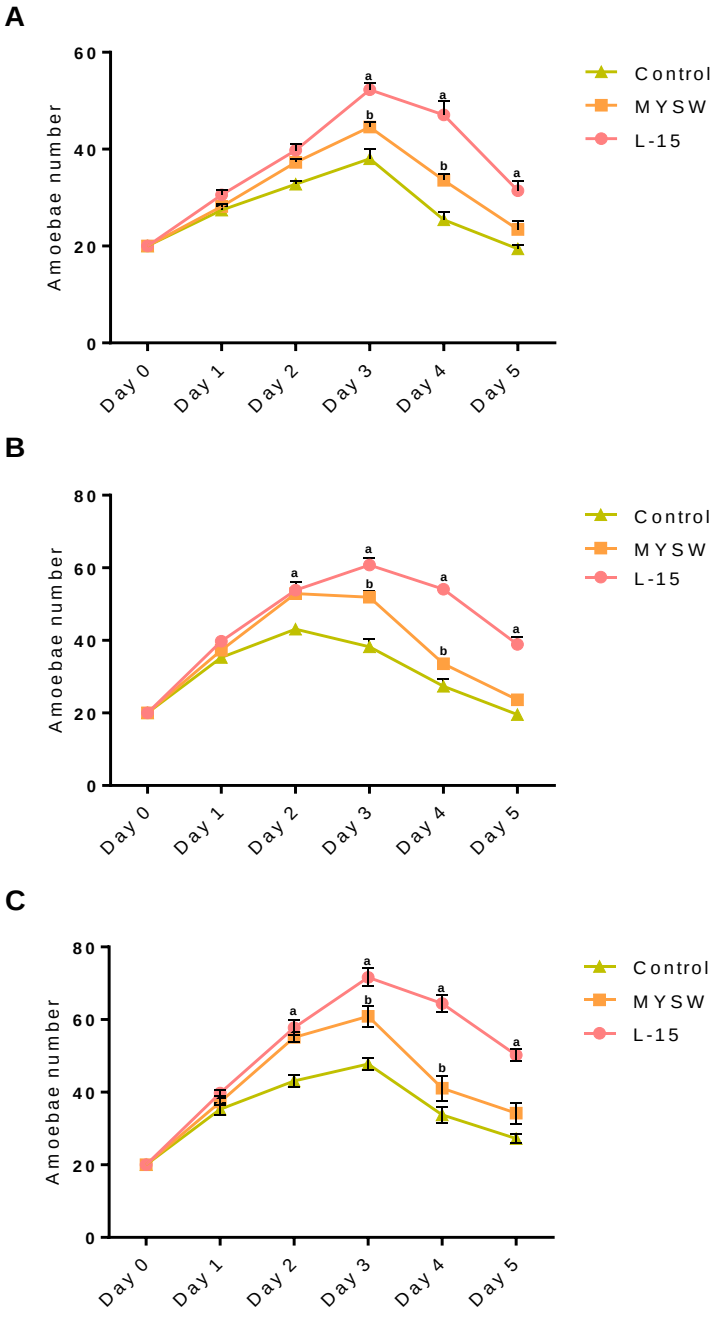

Figure A1.1. Growth curves for *Neoparamoeba perurans* cultured in L-15, malt yeast seawater (MYSW) and colloidal silver seawater as a control over three temperature conditions: 7°C (A), 10°C (B) and 18°C (C). L-15 treatments showed significant numbers ($P < 0.0001$) on the 3rd day (a) and remained significant to the end of the trial. MYSW also showed significant numbers (b)

A1.3.2 TRIAL TWO: ADDITIVES TO L-15 MEDIA FOR ENHANCED GROWTH

Amoebae cultured with FSB and fish mucus showed significantly greater numbers after 3 days in culture when compared with the other treatments (**Error! Reference source not found.**.). However, after day 3, the cultures in FSB acquired a fungal contamination that grew rapidly, and the wells were uncountable due to the contamination by day 6.

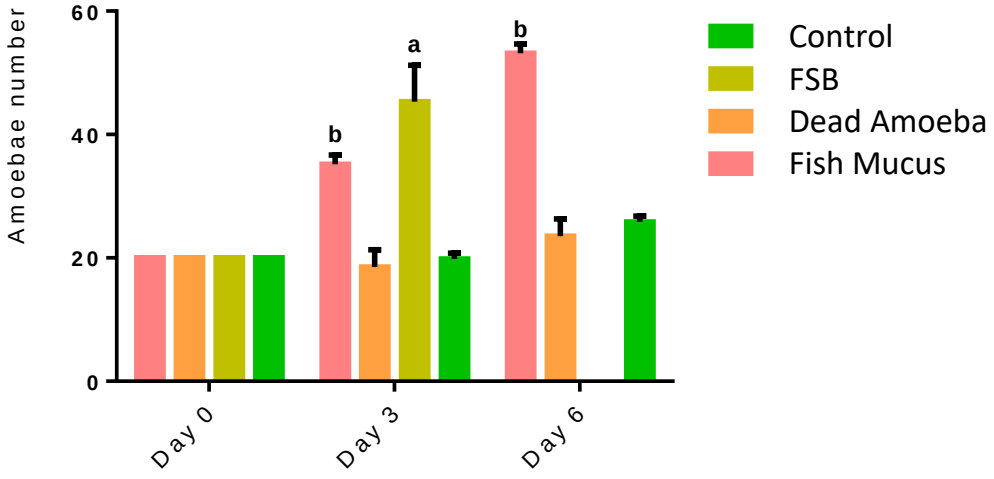

Figure A1.2 Counts of *Neoparamoeba perurans* cultured in L-15 media with the addition of bovine serum albumin (FBS), filtered fish mucus extract, filtered amoebae extract. FBS showed the most significant numbers early (a) (P<0.0001), fish mucus also showed significant numbers when compared to dead amoebae and the control. On day 6, amoebae cultured in fish mucus had significantly higher numbers then the rest of the

treatments. FBS however could not be counted on the final day of the trial due to contamination.

Fungal contamination was also observed in cultures with fish mucus on day 5 of the trail but remained countable. In addition, the wells with fish mucus continued to show increases in amoebae numbers after day 6.

A1.3.3 TRIAL THREE: ANTIBIOTIC ADDITIVES FOR REDUCTION OF BACTERIA TOWARDS AXENIC CULTURES

Over the course of the experiment 5x PSN had the highest numbers of amoebae growth, with both 5x and 6x PSN showing significant amoebae survival ($P<0.05$) (Figure A1.3). Though there was increased growth at 5x and 6x PSN, bacteria were still visible and still growing in the LB for both concentrations two weeks after the final trial day. 10x PSN displayed the quickest visible removal of bacteria, with no bacteria visible on the plate and no growth was seen in the LB media. For media with 8x and 9x PSN no bacteria were visible, and no growth was detected after the 4th day of the trial. In both 10x PSN and 9x PSN the amoebae count remained stable at around 20 cells for the first 3 days (Figure A1.3). This would suggest that treating the cells with a high dose of antibiotics for the first few days in culture may be sufficient to create axenic cultures, then lowering the dose will allow for better growth while preventing new bacterial growth. Though effective on bacterial contaminations, PSN was useless against fungal contaminations. The 2x and 3x PSN treatments developed fungal infection within the first day and by the 2nd day it was impossible to accurately count amoebae cells.

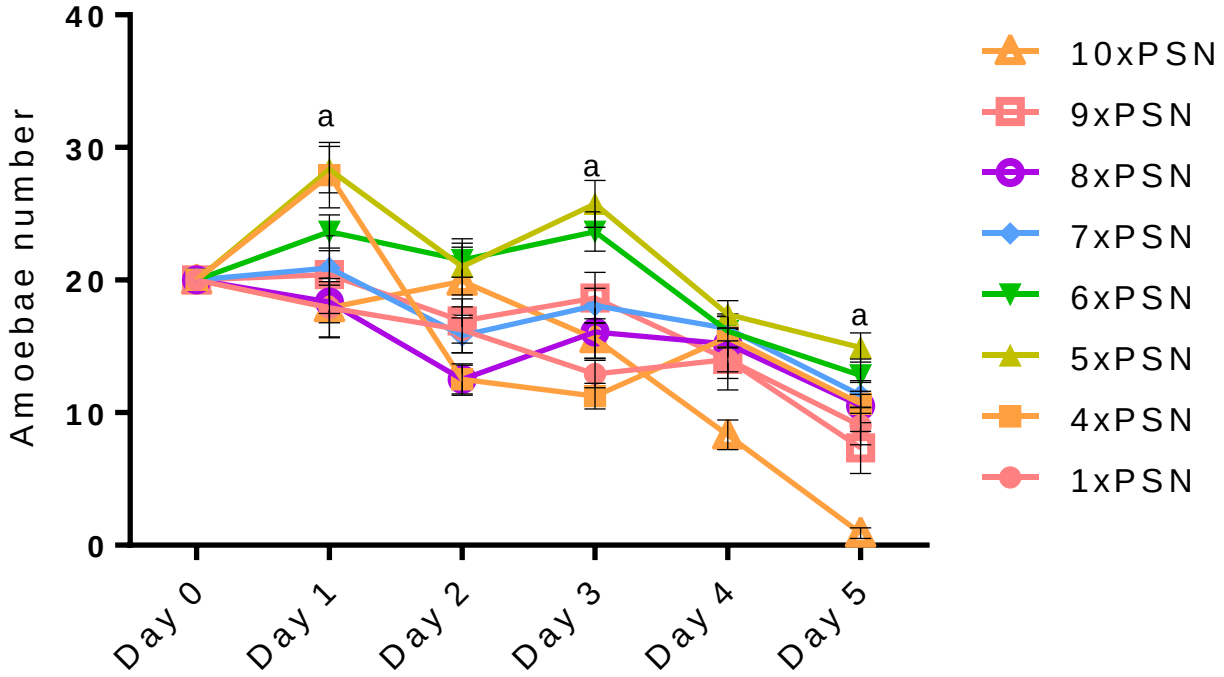

Figure A1.3 Over the course of the 5 days, amoebae in 6x PSN and 5xPSN showed the most sustained numbers. 5x PSN had significantly higher numbers (a) (P<0.0001) on the 1st, 3rd and 5th day. 6x PSN was undistinguishable from 5x PSN from the 2nd day of the trial to its completion. 4x PSN showed significantly better numbers on the 1st day but declined sharply after that. 8x PSN, 9x PSN and 10x PSN were the only wells to achieved axenization, however they exhibited no positive change in numbers and amoebae in 10x PSN were virtually dead by the end of the trial.

A1.3.4 TRIAL FOUR: FISH MUCUS AND FBS CONCENTRATIONS FOR ENHANCED GROWTH

The fourth culture trial compared media with 0, 2.5, 5, and 10% serum concentrations with and without fish mucus. Over the six-day trial period media with 5% and 10% serum only and 10% serum with fish mucus showed significantly higher numbers when compared to all other treatments ($P < 0.05$) (Figure A1.4). Overall, treatments containing the fish mucus showed poor amoebae numbers when compared to the serum. 5% and 10% serum concentrations did not show any significant differences, and this was again confirmed by a further 12 well replication experiment with 5% and 10%.

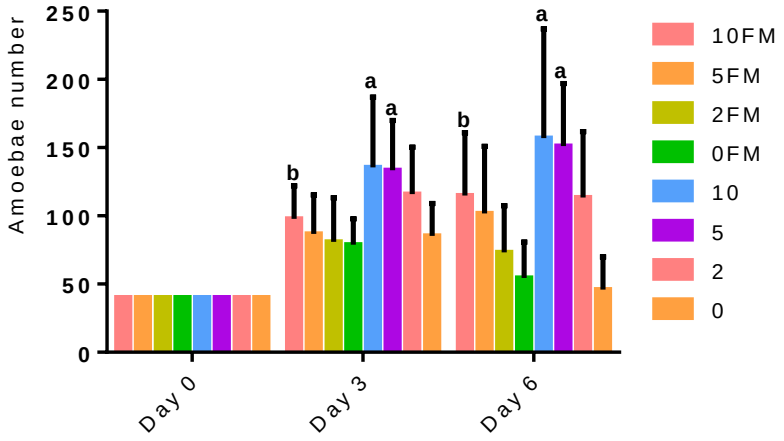

Figure A1.4 *N. perurans* supplemented with serum alone achieved higher numbers better than those supplemented with both serum and filtered fish mucus extract. 10% and 5% serum showed significant numbers (a) ($P<0.0001$) on both the 3rd and 6th days of the trial compared to all other treatments except 10% serum with fish mucus (b). 10% serum and 5% serum were not significantly different from each other.

A1.3.5 SUSTAINABLE LIQUID CULTURES

N. perurans follows a predictable pattern in declining cell morphology towards death as the cultures progress towards an axenic status. It appears that as the limiting reagent is slowly removed from the cultures, the amoebae begin to lift off the surface. Some cells retain partial attachment with long narrow pseudopods visible off the surface and in culture. At a certain point all cells are observed to be floating in suspension, the majority with long narrow pseudopodia. Cell aggregations were also observed at this time. Large clumps from 2 amoebae to 100+ amoebae can be seen floating at various fields of view in the culture flask (Figure A1.5). After this point the cells are no longer seen attaching to the bottom. Growth appears to stop at this point and the majority of cells die. The remaining cells appearing spherical in suspension with no visible pseudopodia and seem to be able to persist in this form for weeks. It is however likely that these forms are pseudocysts that form due to changes in salinity(22).

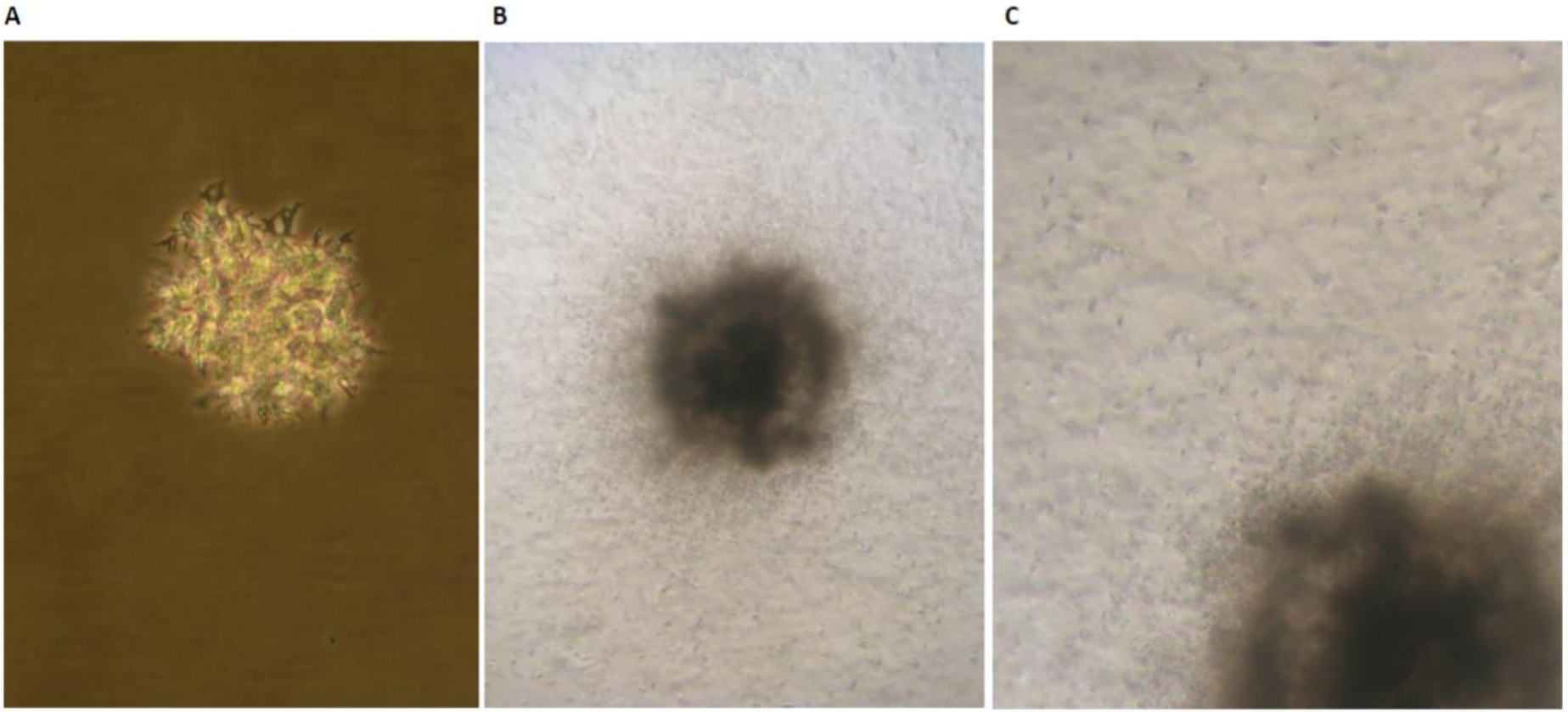

Figure A1.5 A) Amoebae aggregation produced under starvation conditions in liquid culture B) Amoeba aggregation on MYA under starvation conditions visible with the naked eye C Close up of Aggregation showing cells around the edges of the mass

A1.4 DISCUSSION

The fungal and protozoan contaminations were propagated more rapidly at higher temperature and in the wells containing nutritional supplements especially MYSW and FBS. In agar culturing, fungal contaminations can be controlled by the addition of Pimaricin (23). The addition of Pimaricin to liquid cultures in this study however, caused *N. perurans* cultures to die off within a couple days. At present the only way to control a fungal contamination in liquid cultures is constant monitoring and media replacement. This however is tedious, time-consuming and cultures can be unsalvageable. Future studies should investigate other possible anti-fungal agents. In addition, substituting colloidal silver seawater appeared to eliminate flagellate and ciliate contamination over time when combined with the L-15 Media. Temperature also plays a role in the level and probability of a culture succumbing to contamination. Higher temperatures appear to allow for quicker contamination, but lower temperatures slow down amoebic growth. It is therefore recommended that cultures be kept at 10°C as a natural means of contamination control.

Commercially available PSN is an effective antibiotic mixture for the control and eradication of bacteria in cultures. Although axenization can be achieved earlier if treated with 8x PSN or higher, it is recommended that a high initial dose and then continuous treatment with 5xPSN will allow the cells to have the greatest chance to propagate while controlling any possible contamination picked up from subculturing.

As seen with *N. pemaquidensis* (24), *N. perurans* showed the ability to survive and propagate in salt adjusted L-15 media with serum. During the transition to axenic cultures the amoebae appear to grow in the typical stuck down pseudopodial morphology when grown in higher dilutions of L-15 media with glutamax and 10% FCS serum in colloidal

seawater. Successful axenization under the definition of this study was achieved with 8xPSN, 9xPSN and 10x PSN. However, though 'axenic' cultures were established, once the bacteria were no longer visible the cultures began to decline. The cells lost their projections; shape became rounded without crenations and the amoebae lost the ability to attach to the bottom. This would indicate that the cultures were missing an essential compound required for their growth. Future studies should compare common media used for axenic culturing of related marine amoebic species to the recipe for L-15 media to elucidate possible missing components. In addition, future studies should employ a molecular method, such as qPCR, for determining the presence of bacteria if axenization is achieved as this will be the only reliable measure for ensuring pure cultures.

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APPENDIX TWO: JACCARD's SIMILARITY MATRIX FROM CHAPTER THREE

Table A2.1 Jaccard's coefficient similarity distance matrix for the combined RAPD primer profiles (A1, A15, B10, B12 and B18).

	Tas C8	Tas C4a	Tas C4b	Tas C4c	Tas C4d	Tas 1	Tas 2	Tas 3	Norway	Canada	USA	Ireland 1	Ire land 2	Scotland C	Scotland 1	Scotland 2
C8a	**1**															
C4a	**.667**	**1**														
C4b	**.806**	**.719**	**1**													
C4c	**.667**	**.742**	**.774**	**1**												
C4d	**.576**	**.759**	**.625**	**.700**	**1**											
Tas 1	.195	.231	.225	.231	.216	**1**										

Tas 2	.167	.200	.195	.200	.184	**.680**	**1**								
Tas 3	.171	.175	.200	.205	.158	**.708**	**.640**	**1**							
Nor	.250	.289	.282	.324	.353	.265	.229	.200	**1**						
Can	.293	.300	.325	.368	.289	.243	.243	.216	**.516**	**1**					
USA	.149	.128	.174	.152	.111	.343	.343	.278	.200	.342	**1**				
Ire 1	.111	.089	.111	.114	.070	.132	.162	.105	.158	.205	.297	**1**			
Ire 2	.220	.195	.190	.167	.211	.194	.194	.200	.189	.205	.333	.189	**1**		
Scot C	.179	.250	.211	.216	.235	.114	.147	.152	.111	.162	.128	.143	.176	**1**	
Scot 1	.179	.154	.179	.154	.200	.219	.147	.188	.212	.162	.158	.176	.290	.091	**1**

Scot 2	.179	.154	.179	.154	.200	.219	.147	.188	.212	.162	.158	.176	.290	.091	**1**	**1**

APPENDIX THREE: CONCATONATED SEQUENCES FOR MLST ANALYSIS

Table A3.1 Concatenated sequences used in the MLST analysis for each of the samples. The concatenated sequences are 2,425 bp long and include, in order: β-Act, β-Tub, ELF1, ELF2, Rpb1 and SDHA in the 5'-3' direction.

Sample	Concatenated sequence
Tasmania	ACCACCACCGCCGAGCGTGAGATCGTCCGTGACATCAAGGAGAAGCTCTCCTACGTCGCYCTTGACTTCGAGCAAGAGATGCAGACCGCTGCCTCCT
	CCTCTGCCCTCGAGAAGTCCTACGAGCTTCCTGATGGTCAAGTCATCACCATCGGAAACGAGCGCTTCAGGTGCCCTGAGGCTCTCTTCCAACCCTCAT
C8a	TCCTCGGAATGGAGGCTGCTGGCATCCACGAGACCWCYTACAACTCCATCATGAAGTGYGATGTCGACATCCGTAAGGACTTGTACGGCAACGTTGT
	CTTGTCTGGTGGAACCTCCATGTTCCCCGGTATCGCTGACCGCATGCAGAAGGAGTTGACYGCCCTCGCTCCCTCCACCATGCCTTGAAGTTGAACGC
	CCCCACCTACGGAGACTTGAACGGCCTCGTCTCCCAGGTGATGAGCGGTATCACCTGCTCTCTCCGTTTCCCCGGTCAGCTTAACTCTGATCTCCGCAA
	GTTGGCTGTGAACTTGATTCCTTTCCCCCGTCTCCACTTTTTCTTGATCGGGTTCGCCCCTCTCTTCGCCAAAAACGCCCAGCAATACAAGCAACTTTCC
	GTCCAGGAGTTGACCCAGCAGGTGTTCGACAGCAAGAACATGATGGCCGCCACCAACCCCAGGGCCGGACGTTACTTGACCGCCGCCATCACCTTCC
	GTGGCCGTGTCCCCACCAAGGAGGTCGACGACCACCTCTACAAGCTCCCTGGAGGGGGACACGGAGGGGCTTCTCGGAGGGGCGCTTGGGGGGGA
	TGATGTTGTCAAGGGCCTCAAGAAGAGTGGGACCCTTCCACCATCCCATGTTGGTGGATCTCTCCAACATGTTGTCACCGTTCCATCCGGAGATGGGG
	ATGAAGGGGATCTTGTTGGGGTTGTACCCGATCTTCTTCAAGAAACCGGAGACCTCGTTCTTGATCTCAGTGTAACGGTCCTCGGAGTAGTTGACAGA
	CTTGTCGTCCATCTTGTTGATGGCGCAGATCATTTGCTTGACACCGAGGGTGAAGGAGAGAAGGGCGTGCTCGCGGGTCTGACCGTTCTTGGCAATA
	CCAGCCTCGAACTCACCAGTACCGGAGGCAATGACCAAGACGGCAACATCGGCCTGGGAGGTTCCAGTGATCATGTTCTTGATGAAATCACGGTGAC
	CGGGGGCGTCAATGATGGTGAAGTAGAACTTGGAGGTCTCGAACTTCCAGAGAGCGATAGCCAACAAGCACAACCGTCTCTTCTGCAAGGCCGAGC
	CCCTTGACGATGACCTCTCTGTCGAGATCGAGGACGGAAAGATCACCCCCCGTGACGACTTCAAGGCCCGTGCCCGCCACTTGGCCGACACCTACGA
	CTGGAACGTCAACGAGGCCCGTAAGATCTGGTGCTTCGGACCCGACACCTCTGGTGCCAACATCTTGGTCGACTCCACCAAGGCCGTCCAATACTTGA
	ACGAGATCAAGGACTCCGTTGTCGCTGCCTTCCAATGGGCTTCCAAGGAGGGTGTCCTCTGTGACGAGAACATGCGTGGAATCCGCATGAACATTCT
	CGATGTCGCCCTTCACGCTGATGCTATCCACCGTGGTGGAGGTCAGCTCATCCCCACTGCCCGTCGTGTCTTCTACGCTTGCCAGTTGACTGCCGAGCC
	CCGCCTCCAAGAGCCCGTCTACCTTGTCGAGATCCAGTGCCCCGATGTTGCCATTCCGTCTGTTTCTTGAGGTTGTTGTTTGCTTTCACGATGTTGATG

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GAATTTGCTTCTTTTCGACTGTGTGAGAAATGACGTCCTTCAGGTCGGAGTCTTTGTACTCAGCCATGATCCTGAGGCTGTCTTTGGTGATTGTGGTGA
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Tasmania

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C4a

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Tasmania

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C4b

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Tasmania

ACCACCACCGCCGAGCGTGAGATCGTCCGTGACATCAAGGAGAAGCTCTCCTACGTCGCYCTTGACTTCGAGCAAGAGATGCAGACCGCTGCCTCCT
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C4d

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APPENDIX FOUR: PAPERS PUBLISHED DURING PHD CANDIDATURE

1. **Jessica Johnson-Mackinnon**, Tina Oldham, and Barbara Nowak. "Amoebic Gill Disease: A Growing Threat." Microbiology Australia Microbiol. Aust.37.3 (2016): 14042.

Abstract: The risk of disease outbreaks is predicted to increase due to climate change. For farmed fish an example is amoebic gill disease (AGD). While initially reported only in farmed salmonids in Washington State, USA, and Tasmania, Australia, it has now become an issue for Atlantic salmon farming worldwide and affects a range of other farmed marine fish species. Local high temperature anomalies and a lack of rainfall have been associated with the outbreaks of AGD. This worldwide presence is at least partly due to the cosmopolitan nature of the parasite and its low host-specificity. The disease can be treated using freshwater or hydrogen peroxide baths, but the treatments increase the cost of salmon production. Management of AGD contributes 20% to production costs of Atlantic salmon in Tasmania

2. Goro Tanifuji, Ugo Cenci, Daniel Moog, Samuel Dean, Takuro Nakayama, Vojtěch David, Ivan Fiala, Bruce A. Curtis, Shannon Sibbald, Naoko T. Onodera, Morgan Colp, Pavel Flegontov, **Jessica Johnson-MacKinnon**, Michael McPhee, Yuji Inagaki, Tetsuo Hashimoto, Steven Kelly, Keith Gull, Julius Lukeš, and John M. Archibalda, Genome

sequencing reveals metabolic and cellular interdependence in an amoeba-kinetoplastid symbiosis. Scientific Reports, 2017; 7: 11688

Abstract: Endosymbiotic relationships between eukaryotic and prokaryotic cells are common in nature. Endosymbiosis between two eukaryotes are also known; *cyanobacterium*-derived plastids have spread horizontally when one eukaryote assimilated another. A unique instance of a non-photosynthetic, eukaryotic endosymbiont involves members of the genus *Paramoeba*, amoebozoans that infect marine animals such as farmed fish and sea urchins. *Paramoeba* species harbor endosymbionts belonging to the Kinetoplastea, a diverse group of flagellate protists including some that cause devastating diseases. To elucidate the nature of this eukaryote-eukaryote association, we sequenced the genomes and transcriptomes of *Paramoeba pemaquidensis* and its endosymbiont *Perkinsela sp*. The endosymbiont nuclear genome is ~9.5 Mbp in size, the smallest of a kinetoplastid thus far discovered. Genomic analyses show that *Perkinsela sp.* has lost the ability to make a flagellum but retains hallmark features of kinetoplastid biology, including polycistronic transcription, trans-splicing, and a glycosome-like organelle. Mosaic biochemical pathways suggest extensive 'cross-talk' between the two organisms, and electron microscopy shows that the endosymbiont ingests amoeba cytoplasm, a novel form of endosymbiont-host communication. Our data reveal the cell biological and biochemical basis of the obligate relationship between *Perkinsela sp.* and its amoeba host and provide a foundation for understanding pathogenicity determinants in economically important *Paramoeba*.